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AGRICULTURAL RESEARCH INSTITUTE
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SERIES B

CONTAINING PAPERS OF A BIOLOGICAL CHARACTER

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PROCEEDINGS OF

THE ROYAL SOCIETY.

Section B.—Biological Sciences.

Address of the President, Sir Ernest Rutherford, at the Anniversary Meeting, November 30, 1926.

At this anniversary meeting, it is customary to refer to the losses by death suffered by our Society during the year. These include sixteen of our Fellows and one Foreign Member. The Society has to lament the untimely removal of some of our most distinguished workers in the field of general Biology and Pathology.

On January 2 died John Gray McKendrick, Emeritus Professor in the University of Glasgow, in his eighty-fifth year. A veteran Physiologist, he collaborated with the late Sir James Dewar in the discovery of the excitation by light of an electric current in the retina and optic nerve, and made distinguished contributions to the physiology of the special senses. He was a man of wide interest, distinguished also as a lecturer and writer.

Benjamin Neeve Peach, aged 84, was for many years a member of the Geological Survey of Scotland. Pre-eminent as a field geologist, both by his teaching and original contributions, he influenced greatly the progress of Scottish Geology.

The untimely death of William Bateson has deprived the Society and Natural Science of a great biologist. As the immediate sequel of a brilliant academic career came studies that gained for him an outstanding position as a comparative anatomist. These studies proved only a prelude to an exhaustive investigation of variation in species, which revealed in him the qualities of a scientific observer and traveller of the first rank.

The sustained study of the principles of heredity, to which his energies were now devoted, enabled him to found and to inspire at Cambridge a genetical school whose discoveries have added materially to natural knowledge. The last fifteen years of all too short a life were given by Bateson, as Director of the Horticultural Institution at Merton, to a continuation of the pursuits initiated at Cambridge, to the application of results to culture, and to the establishment of co-operation between the practical breeder and the scientific student. Bateson was more than the foremost exponent of Mendelian research in this country. A highly skilled amateur of art, with a special knowledge of the art of the Far East, he was an ardent and successful collector. Among the specimens his taste and knowledge enabled him to acquire, which now serve as a permanent memorial of him and of his association with the British Museum, are things, their present custodian assures us, which the national collection "could hardly have hoped to acquire by purchase." The happy combination of penetrating vision, balanced judgment and generous readiness to comply with requests for counsel which enabled Bateson to do the State the service of fostering a harmonious relationship between science and practice in breeding, was equally marked in all that he did for the Society as a member of many important Committees.

JAMES FAIRLIE GEMMILL, in later years Professor of Natural History, University College, Dundee, and formerly teacher in the University of Glasgow, was a Zoologist with a special interest in Embryology. He helped much by his devotion and example to develop the Millport Biological Station as a centre of training and research.

By the death in his seventy-third year of HEIKE KAMERLINGH ONNES, Foreign Member and Rumford Medallist of our Society, and Nobel Laureate, we have lost one of the greatest experimenters of our age. Influenced by the teaching of his great countryman, van der Waals, he early decided to devote his life to the experimental study of the properties of matter at low temperatures. By his power of organisation and his experimental skill, he built up in the University of Leyden the Cryogenic Laboratory, which is unique in the world. His most spectacular triumph was the liquefaction of helium in 1908, but of still greater scientific importance was his fundamental discovery of the electrical supra-conductivity of certain metals at temperatures closely approaching the absolute zero. It is difficult to exaggerate the significance of this discovery, which is likely ultimately to provide the key to the explanation of the passage of electricity through metals. Science owes him a debt, not only for his personal contributions to knowledge, but for his international services in offering freely the facilities of his laboratory to other workers on the effects of low temperatures. No one could fail to be attracted by his genial and forceful personality.

ARTHUR ROBERTSON CUSHNY, Pharmacologist, recognised as a leader in that department of medical science throughout the world. Having been assistant to Schmiedeberg, one of the founders of the modern science of experimental pharmacology, Cushny was for 13 years Professor in that subject in the Medical School of Ann Arbor, Michigan, for 12 years in University College, London, and for the last eight years of his life in the University of Edinburgh. Among his many original contributions to science may be mentioned, his experimental analysis of the causes of irregularity in the action of the heart, and of the remedial action of digitalin and similar drugs in heart disease ; his demonstration of the wide difference in physiological activity between optical isomers ; and his work on the mechanism of the secretion of urine. He was a man of deep learning and wise judgment in all matters pertaining to the subject of his life's work, and his opinion and advice were sought and trusted in scientific and administrative circles alike.

SIR PHILIP WATTS, who died in his eightieth year, was for the greater part of his active life associated with the development of Naval Architecture in the Admiralty. From 1885 to 1912 he was in charge of the design of warships in Armstrong's works, and in 1902 was appointed Director of Naval Construction in the Admiralty. When Lord Fisher became First Sea Lord, Watts was given his opportunity to produce the big-gun ship. The combination of the high technical skill of Watts with the unbounded energy of Lord Fisher resulted in the production of the series of Dreadnoughts and Super-Dreadnoughts of which our Grand Fleet was mainly composed when the hour of battle came. A man of marked scientific ability, he contributed many papers of high merit. A valued member of our Society, he served on its Council and was Vice-President in 1915.

WILLIAM JAMES LEWIS, who died in his eightieth year, was for 45 years Professor of Mineralogy in the University of Cambridge. A mineralogist of the old school he had many interests, ranging from the collection of mineralogical specimens to the direction of a scholastic agency which he founded as a private venture in 1884, long before the setting up of an official Appointments Board by the University.

HENRY BROUGHAM GUPPY, already endowed with the instincts of a naturalist took ample advantage of the opportunities enjoyed by a naval surgeon for observation in the field, and devoted to this work the years that followed his retirement from public service. His death took place during his return from a visit to the Pacific undertaken in connection with his scientific studies.

JAMES THOMSON BOTTOMLEY, a nephew of the Lord Kelvin, was closely associated with him not only in the Physics Department of the University of Glasgow but also in the design and construction of scientific instruments.

An active experimenter in many branches of Physics, well known as the author of a useful compilation of four figures mathematical Tables.

WILLIAM BOOG LEISHMAN, Lieutenant-General, since 1923 Director-General of the Army Medical Service, distinguished for his practical application of immunology to the protection of the Army, during peace and in the field, and for his brilliant original discoveries in the pathology of tropical disease. Leishman was associated with Almroth Wright at a time when the method of protective inoculation against the enteric fevers was being evolved, and had later the chief responsibility for organising and perfecting the measures for its practical application on the largest scale. A measure of his success in this application of scientific principles was given, in due course, by the relatively trivial proportion of deaths from the enteric fevers in the British armies during the great war. Leishman discovered in 1900, though he did not publish the discovery until 1903, the protozoal organism responsible for Kala Azar, thus opening the way for the later discovery by others of the means of treating this widely spread endemic fever of eastern lands, formerly regarded as inevitably fatal. The generic name Leishmania, given to this and allied organisms, commemorates his association with the discovery. In more recent years he was engaged in brilliant and suggestive observations on the developmental cycle in its intermediate host, a tick, of the *Spirochœta duttoni*. This work was interrupted by calls made, during the war and since its close, on his administrative ability and experience. He was Pathological Adviser to the Expeditionary Force in France, became Director of Pathology to the Army Medical Department in 1919, and in 1923 Director-General of the Service to which he was to give but three more years of a devotion too eager for his strength. Among his many activities he found time for good service to the Royal Society, on its Council and its Committees, and was a Vice-President from 1921 to 1922. His untimely death has brought a heavy loss to science and the nation.

FREDERICK WALKER MOTT, neurologist, working both as a pathologist and clinical investigator, made contributions of great importance to knowledge of the causes, and of the means of treatment, of diseases of the brain and the nervous system. For 28 years Mott held with great distinction the position of Pathologist to the London County Asylums, and his laboratory at Claybury became a centre of progressive research for visiting neurologists of this and other countries. Among his more important contributions to science may be mentioned the definite discovery that general paralytic insanity is due to a specific infection, and therefore no longer a hopeless problem from the point of view of either prevention or treatment; his demonstration that the so-called

" asylum dysentery " is a bacillary dysentery occurring and spreading under the conditions of asylum life, so that it can be eliminated by adequate sanitary measures ; his work on the pathology of *dementia præcox,* and on heredity in mental and nervous diseases.

THE REV. THOMAS ROSCOE REDE STEBBING died aged 91. At one time master in our Public Schools and then Fellow and Tutor of an Oxford College, in mature age, attracted by the writings of Darwin, he threw himself into the controversy then raging in support of the theory of evolution. To gain first-hand knowledge, he studied the Crustacea, and was entrusted with the examination of the material obtained in the Challenger Expedition. His masterly report covering three volumes was admirable in arrangement and in literary form. Trained in the older learning, he brought to the study of science not only much erudition but a sense of the historical perspective of knowledge.

JOHN GEORGE ADAMI, pathologist, Vice-Chancellor of the University of Liverpool since 1919, and before that for 17 years Strathcona Professor of Pathology in McGill University, Montreal. Early associated with the late C. S. Roy in pioneer research on the borderland between physiology and pathology, Adami was later responsible for many original contributions to science, in a wide field of pathological investigation, and author of a well known and comprehensive work on the Principles of Pathology. He served during the war as Assistant Director of the Canadian Army Medical Corps, and in more recent years, gave distinguished service to the cause of general and medical education. A man of wide interests and sympathies, he showed a genius for friendship.

In the early death of FREDERICK WILLIAM GAMBLE, at the age of 57, Zoölogy has lost a distinguished teacher and investigator. At one time lecturer in the University of Manchester and then Professor of Zoology in the University of Birmingham, he proved himself an inspiring lecturer and an enthusiastic investigator. Some of his most important work in conjunction with Sir Frederick Keeble was connected with the discovery of the colour changes of the " Phantom shrimp," and other crustacea, and the working out of their physiological significance. His two small books " Animal Life " and the " Animal World " are admirable examples of the presentation of the problems of biology to an unscientific audience.

JOSEPH HENRY MAIDEN, of English birth and Australian adoption, applied with sound judgment for 45 years a wide and accurate knowledge of botany to the furtherance of the economic interests of the great Commonwealth in which he lived, and to the advancement of science and education in Australasia.

On October 9, died GEORGE WILLIAM LAMPLUGH, aged 67, for long a member of the Geological Survey of this country and in later years an Assistant Director. A geologist of wide experience and a traveller in many lands, he made numerous contributions to our knowledge of the geology of Great Britain and Ireland. He was an active member of our Society serving on its Council, 1914–16.

Turning to other matters, we have to record a gratifying improvement in connection with international scientific relations. For two successive years, the Council of our Society had unanimously supported the admission of the Central Powers to full membership of the International Research Council. At the meeting in Brussels this summer, I had the honour of moving a resolution in this sense which was carried unanimously. It is understood that this invitation to join the International Research Council will be accepted in due course. In achieving this happy result, we owe much to the wise guidance of Sir Richard Glazebrook, our Foreign Secretary, and to Sir Arthur Schuster. I am sure that all members of our Society will welcome the disappearance of restrictions in membership which were seriously hampering the international co-operation of scientific men. We may look forward hopefully to an ever-growing friendship and co-operation between the scientific men of different nations, thus promoting not only the advance of science but goodwill between the nations.

It is a great pleasure to me to record the gift from one of our Fellows, Mr. Campbell Swinton, of £1,000 for a fund to be used for other than direct scientific purposes. The donor in his letter to the Council, expressed the hope "that others may be induced to subscribe to the fund so that the income may in time become sufficient for the purpose for which, as I understand, no specific fund is available." In attendance at the meetings of our Council and Committees, some of our members who come from a distance often incur heavy expenses which have been uncomplainingly borne. A fund to be used for this and other general purposes of the Society would be of much help.

I would like to take this opportunity to refer briefly to the striking advances in radio-communication which have been made this year, and to the new avenues of research in the electrical state of our atmosphere that are being opened up by the study of the mode of propagation of wireless waves over the earth.

Among the many developments of science during the past 30 years, none has left a deeper impression on the lay and scientific mind alike than the remarkable growth of wireless as a means of long-distance transmissions of signals, speech, music and even of pictures. The history of this new method of signalling is of special interest to all scientific men, for it illustrates in a vivid way the value of a close co-operation between pure and applied science for rapid progress. The first great chapter in the history of radio-communication we owe to the genius of Maxwell, who, in a paper communicated to this Society in 1864 entitled "A Dynamical Theory of the Electromagnetic Field," showed that electric and magnetic effects cannot be produced instantaneously at a distance, but must be propagated through space with the velocity of light. He demonstrated the wave-nature of these electrical disturbances in space and the mode of their propagation. It is no exaggeration to say that the complete theory of electrical waves and their transmission in space is contained in his famous equations, and that too at a time when no experimental methods were known of producing or studying such electrical waves.

The next great step in advance we owe to the brilliant researches of Hertz, who in 1887, in his laboratory at Karlsruhe, showed how electrical waves in space could be produced by an open electric oscillator, and devised methods for their detection and study.

It was not long before the results of these small scale laboratory experiments were applied for practical ends. In 1896, attempts began to be made in England to utilise electric waves for signalling purposes, and the rapid development of this new branch of applied science owes much to the pioneer work of Marconi and Lodge. Progress in the later stages has been largely influenced by the utilisation of another scientific discovery, namely the use of electric currents in vacuum tubes as a powerful method of producing and detecting electrical waves. It is of interest to note that the first use of an electron tube as a detector of electrical waves was made by Professor J. A. Fleming.

It is not my object to detail subsequent progress except to refer to the noteworthy developments that have taken place this year. On January 1 the new Post Office Station at Rugby was opened for the transmission of messages to ships in all quarters of the globe and for Foreign Office messages. This long wave installation is in many respects unique. It is the only high-power electron-tube station in the world, and contains many novel features in its design and operation. The frequency of the continuous waves emitted by this Station is controlled with great accuracy by a vibrating tuning fork and the numerous high-power electron tubes in parallel are used to magnify five hundred thousand million times the energy of one of the harmonics of a small triode valve operating the tuning fork. The extraordinary flexibility of these electron tubes both as oscillators and receivers has been fully utilised in the design of the installation which incorporates all the latest developments in this branch of science. The success of the station for the purposes for which it was designed is a tribute to the breadth of the scientific knowledge and the boldness of the initiative displayed by the Imperial Wireless Commission and the Engineering staff of the Post Office. This station has been also successfully used for experiments in radiotelephony with the Long Island station near New York. It is now possible, and will, it is expected, soon be practicable, to connect any telephone subscriber in Western Europe with any telephone subscriber on the North American Continent.

During the past two or three years, telegraphy on a commercial scale by the aid of short waves has been conducted in many countries. A still further development by the Marconi Company is to be recorded this year. Short wave stations, in which a series of parallel wires are arranged to act as a reflector and emit a beam of waves in a definite direction, have been erected near Bodmin in Cornwall and near Montreal in Canada. After a successful series of experiments, these stations began to operate commercially last month. Similar stations for communicating with the other Dominions are in the course of erection. It will be of great interest to see how far a continuous service is feasible by these new methods, in spite of the atmospheric disturbances which sometimes so seriously affect ordinary short wave transmissions.

It is remarkable how the progress of applied science in many instances depends on the utilisation of some obscure property of matter discovered in the course of purely scientific experiments. For example, in 1888, the brothers J. and P. Curie, working on the properties of crystals, discovered the piezo-electric properties of quartz. In a suitably cut crystal of quartz, an electric charge on the surface appears when the crystal is compressed or extended. Conversely a charge applied to the surface of the crystal alters its dimensions. No one at that time could have foreseen that this property could be utilised to control, automatically and with great accuracy, the frequency of the waves emitted by Broadcasting Stations, and thus be a factor of great importance in reducing interference between stations. Illustrations of this kind can easily be multiplied. For example, the discovery about 30 years ago in the laboratory of the photo-electric effect, in which certain substances exposed to light produce a copious emission of electrons, has formed the essential basis of the methods used to-day in transmitting radio-pictures and in experiments on tele-vision.

I should like to add a few words in connection with the problem of the propagation of long and short electrical waves over great terrestrial distances, which has been the subject of discussion for many years, but on which valuable new data have been recently obtained. When wireless signals were first transmitted across the Atlantic, the late Lord Rayleigh immediately raised the question whether the waves were able to follow the curvature of the earth by the agency of diffraction alone. This problem has attracted the attention of many able mathematicians who have shown conclusively that some other agency must enter into the transmission of these waves over great distances. It was early suggested by Kennelly and Heaviside that the bending of the rays might be accounted for by supposing that there was in the upper atmosphere a layer which was electrically conducting and which guided the waves round the earth's surface. Precision was given to this view by the work of Eccles and others, who showed that ionised gases could refract and absorb electrical waves passing through them. A still further advance was recently made when Sir Joseph Larmor pointed out the paramount importance of the long free path of the electrons in the upper atmosphere in producing scattering and refraction of electrical waves. He showed that a comparatively sparse distribution of electrons was sufficient to bend the path of the rays round the earth.

A direct attack on this problem has been recently made in this country by several methods, and convincing evidence has been obtained of the existence and height of this refracting layer. Appleton and Barnett, using wave-lengths of about 400 metres, have shown that at moderate distances from a wireless transmitter two sets of waves are received which produce interference phenomena. One set of waves travels in a straight line from the transmitter along the ground and the other passes into the upper atmosphere where it is refracted or reflected back to the receiving station. These experiments are of much interest as providing large scale analogues of the ordinary optical interference experiment carried out with wave-lengths a thousand million times as long. From the results of these investigations the height of the effective layer is estimated to be about 90 kilometres. In general, the refracted ray is elliptically polarised, an effect no doubt connected with the action of the earth's magnetic field on the motion of the free electrons. Similar results by other methods have been obtained by Rose and Barfield of the National Physical Laboratory. These important series of experiments have been conducted in connection with the Radio-Research Board of the Department of Scientific and Industrial Research, and it is a pleasure to me to welcome here to-day its Chairman, Admiral Sir Henry Jackson, as one of our Medallists.

These observations not only give an explanation of night-time errors in direction-finding and of signal variations, but in a general way throw light on the vagaries observed in the transmission of short waves, where a signal may be undetectable a few miles away from the transmitter but may be received strongly a thousand miles away.

While the study of the propagation of electrical waves through our atmosphere is of much interest in itself, it is of even more value as giving us a new and powerful method of attack on the problem of the electrical state of our atmosphere, particularly at heights where direct observations are impossible. We may anticipate that an extension of such experiments will provide us with much valuable information not only on the degree of ionisation of the upper atmosphere but on its diurnal and seasonal variations. Although only preliminary observations have so far been made on this question, the results obtained show that there is much promise in this new method of attack on a difficult problem.

The phenomena of the aurora and the diurnal variation of the earth's magnetism have long been supposed to indicate that the upper atmosphere is highly ionised and an excellent conductor of electricity. The origin of the ionisation is a matter of much interest. Part, no doubt, is due to the ultra-violet light emitted by the sun, but there may be other important contributary causes. During this year, E. A. Milne has shown how certain atoms of matter, ejected from the sun, notably those of calcium, may, in consequence of absorption and emission of radiation, acquire sufficiently high velocities to penetrate deeply into our atmosphere. It may be that the brilliant auroræ and magnetic storms which so often accompany sunspot activity, are a consequence of the projection into our atmosphere not only of electrons, as has long been supposed, but of swiftly moving atoms of matter.

Another source of ionisation to be taken into account is the very penetrating radiation in the upper atmosphere brought to light by the experiments of Kohlhörster and Millikan. The origin and nature of this radiation is still *sub judice*. Some have supposed it to be of cosmical origin and see in it evidence of the disintegration or formation of atoms of matter in worlds remote from us. On the other hand, we must not exclude the possibility of a mundane origin, for C. T. R. Wilson has given very strong reasons for believing that very high speed electrons and penetrating radiations may be produced as a result of the movement of electrons in the intense electric fields which arise during thunderstorms. This penetrating radiation has been detected by the minute ionisation observed in electroscopes at high altitudes. The effects are very small and the experiments difficult, but we may hope to obtain more definite information as to the origin and nature of this radiation by the experiments now in progress.

I now pass to the presentation of medals.

The Copley Medal is awarded to Sir Frederick Gowland Hopkins.

For 20 years, Sir Frederick Hopkins has been a foremost leader in Biochemistry, a branch of science that has grown rapidly in importance and influence during this period. The guide and Director of a great Research Laboratory in Cambridge, he is everywhere recognised as one of the great pioneers of his science. In his active life, he has made a series of fundamental discoveries, each of which has led to the opening up of new fields of work. The isolation and identification of tryptophane 25 years ago, at a time when but few of the amino-acids that enter into the composition of proteins were recognised, marked an epoch in the pure chemistry of these substances. The importance of this discovery was enhanced by Hopkins' later work on this substance, which led to a revolution in the physiology of proteins in nutrition, the end of which is not yet in sight. Some of the most fruitful work in recent physiology has been upon the nature of muscular contraction. The work of A. V. Hill and of Meyerhof, of Embden, and many others, turns upon the fundamental earlier discoveries by which Hopkins, in collaboration with W. M. Fletcher, defined the conditions governing the appearance of lactic acid in muscle during activity, and its disappearance during recovery. One of the most important discoveries of this century is summed up in the word Vitamines. Fifteen years ago, Hopkins had carried out experiments which not only showed that appropriate mixtures of proteins, carbohydrates, fats and salts, might, for lack of traces of unknown substances, be inadequate for the nutrition of animals, but at the same time established the general lines of the methods used ever since in the investigation of these substances by important groups of biochemists in all parts of the world. The discovery of the dipeptide glutathione, coming at a time when the nature of the processes underlying biochemical oxidations was the subject of significant work in many laboratories, has again brought Hopkins into the van as a leader in yet another part of the field of biochemistry and given the signal for intense renewed activity there. Hopkins' work throughout has shown a genius for discovery. It has inspired a very large part of the best work in biochemistry in this century.

The Rumford Medal is awarded to Sir Arthur Schuster.

Sir Arthur Schuster began his work on Optics in the early days of Spectrum Analysis, and, indeed, was the first to employ in 1881 the word Spectroscopy to designate this branch of Science. He has made numerous original contributions to Optics in many directions. We may refer particularly to his work in the group velocity of waves and the pulse theory of white light. The breadth and penetration of his knowledge is clearly shown in his book on "Optics," a standard work which has served as a guide for generations of students. Schuster has made valuable contributions to many branches of Experimental and Mathematical Physics. When Professor in the University of Manchester, he made with Gannon a well-known determination of Joule's equivalent, and did valuable work in that connection by calibrating the thermometer originally used by Joule in his fundamental experiments. He was a pioneer in the study of the discharge of electricity through gases, and has taken a deep interest in the problems of Geophysics, particularly in connection with the magnetism of the earth and the state of the upper atmosphere, and is responsible for valuable additions to knowledge in these fields. Besides this original work, Schuster has always shown an active and keen interest in the progress and organisation of Science. He was a member of the General Board of the National Physical Laboratory from its inauguration and for six years acted as Chairman of its Executive Committee. He was Secretary of our Society 1912–19 in the difficult war period and Foreign Secretary 1920–24. He took an active part in the formation of the International Research Council, and since its inception has acted as its Secretary. The Society owes much to his wise guidance and to his generosity.

A Royal Medal is awarded to Sir William Bate Hardy.

The scientific investigations of Sir William Hardy in Physical Chemistry and Physics are of outstanding importance in many different fields of work, and are characterised by the highest degree of originality. In colloid chemistry, his name is known in every country for the fundamental and pioneer work which he has accomplished in that field. The stability of colloid sols in relation to the electric charge, the theory of flocculation, the nature and importance of the iso-electric point, the theory of protein ampholytes, and the electric charges of the positive and negative colloid ions represent some of the important discoveries with which his name will be for ever associated. The modern theory of protein solutions, which is of such great importance in biochemistry and physiology, is very largely due to his pioneer work in that field.

Sir William Hardy has also been a pioneer in the elucidation of the nature of surface forces and surface films and the orientation of molecules at surfaces. This work has been of the highest importance for the development of a new and extremely important branch of physico-chemical science. As a natural outcome of this work, Sir William Hardy has turned his attention in recent years to the friction between surfaces and the nature of lubrication, and in a series of important investigations has thrown a flood of light on a subject which had long been neglected by both physicists and chemists. For the first time in the history of science the dependence of friction and lubrication on the structure and molecular orientation of surface films and the force-fields of molecules in relation to their structure and polarity have been elucidated in a series of beautiful and highly important researches.

In all these various fields of molecular science—colloid chemistry and physics, surface films and molecular orientation, friction and lubrication—the researches of Sir William Hardy have been of fundamental importance and have been characterised by an originality of outlook and an imaginative insight which bear the impress of genius.

I can only refer in passing to his many original contributions to another branch of Science, Physiology, and to the work that he has done for Science and Industry as Director of the Low Temperature Laboratory at Cambridge. For many years one of our Secretaries, we are grateful for his devoted service.

A Royal Medal is awarded to Professor Archibald Vivian Hill.

Professor A. V. Hill has made important contributions to knowledge of muscle and nerve. As to the former his enquiries, begun some 16 years since, were taken up at a time when, owing to the emergence of new facts, views of general acceptance stood in essential need of re-examination. In the past seven years Hill has accomplished this with a success beyond expectation. He has related to the mechanical the thermal aspects of muscular activity with a precision hitherto unattained, and obtained data as valuable for the chemical as they are fundamental for the physical study of the problem. The technique developed by him enabled for the first time the discrimination, in the heat production of muscle, of successive quantities and rates characterising successive stages of that activity, in spite of the closely consecutive and in part evanescent character of those phases. "Initial heat," uninfluenced by oxygen, the immediate accompaniment of the mechanical changes in the muscle, was thus distinguished from a "delayed heat" associated with functional recovery of the muscle; and in this latter there were recognised two portions which evaluate the relative shares of aerobic and anaerobic disappearance of lactic acid in the processes of restoration of the muscle. In association with this recovery process the molecular ratio between removed and oxidised lactic acid has thus been estimated. Besides furnishing this essential analysis of the functional reactions of isolated muscle, Hill has prosecuted notable enquiries into the factors conditioning the performance and maintenance of muscular effort in the human body, measured its chemical cost and traced to their causes certain of the limits set to the speed and endurance of the athlete. Further, Hill has succeeded in not only detecting but in measuring heat-production accompanying the conductive activity of nerve. The scale of energy-change involved in this has required the devising of a refined technique; here again he with his pupils has obtained and measured the heat not only in block but in its separate phases of production. Both for muscle and nerve, the advances made by Professor Hill and his collaborators place knowledge on a new footing; whenever the intimate mechanism of the activity of these two important tissues may finally be elucidated, it is certain that the contributions of Professor Hill will remain fundamental for the explanation of the mechanism of them both.

The Davy Medal is awarded to Sir James Walker.

The investigations of Sir James Walker in the field of physical chemistry have been of great importance in the advance of that science. His work on the electro-synthesis of organic compounds, carried out originally in conjunction with the late Professor Crum Brown and continued up to the present time, has thrown much light on the phenomena of electrolysis and has led to the synthesis of a large number of new and interesting substances. One of the pioneer investigators of ionic equilibria he has developed the theory of Arrhenius in many directions. His discovery of the nature and equilibria of amphoteric electrolytes constitutes a fundamental advance which has been of the greatest fruitfulness not only for the general theory of electrolytic solutions, but also for the development of the chemistry of the proteins. The work of Sir James Walker on the theory of amphoteric electrolytes represents one of the greatest advances in the elucidation of the nature of solutions. His investigations on the adsorption of dissolved substances by solids, for example, the adsorption of picric acid by silk fibres, were of cardinal importance in the recognition of the true nature of a class of phenomena whose occurrence has been established in many branches of science. In the theory of reaction-velocity and chemical reactivity, and in many other parts of physical chemistry, the work of Sir James Walker is of high value and importance.

The Darwin Medal is awarded to Dr. Dukinfield Henry Scott, for his distinguished work on Fossil Plants.

At a moment when there seemed some danger that the brilliant advances in Palæophytology made by Professor Williamson might slacken owing to advancing years, Dr. Scott entered upon a fruitful co-operation with the veteran. Several joint Memoirs were the result of this happy coalition; but later Scott established a quite independent position of his own. Among the numerous Memoirs published by him during the last 40 years, none stands out more prominently as a model of presentation of complex structure than that on *Cheirostrobus*, a new type from the Calciferous Sandstone. Not only was its elaborate structure fully described, but the comparative treatment showed a master hand.

This quality came out with even greater effect in the study of the new class of the Pteridosperms, or primitive Seed-Plants with Fern-like habit. The extensive knowledge of these early land-plants which we now possess has been mainly based upon the work of Scott, Oliver and Kidston.

Such work, of which these examples do nothing more than suggest the nature and the scope, has been gathered up by Scott into his "Studies in Fossil Botany," a book now in its third edition. It deals primarily with early Vascular Plants, placing them in natural relation to their living correlatives, and giving a picture of early land-vegetation that has never been surpassed in clarity of presentment, combined with accuracy of detail and of reference. This constitutes a real constructive contribution to natural knowledge. It supplies not only a great mass of fact that is positive and new; but it also subjects those facts to a detailed criticism and a philosophical treatment such as Darwin himself would have been among the first to appreciate.

The Hughes Medal is awarded to Admiral of the Fleet Sir Henry Bradwardine Jackson, in recognition of his investigations in Radio-Telegraphy.

His experiments date from 1899, and in the following year apparatus of his design was fitted to certain of H.M. ships, and some of the problems connected with the screening effects of high land were investigated by him. He described his results in a paper read before the Royal Society on May 15, 1902. At a later date, he was responsible for the erection of one of the earliest continuous wave stations in which an arc of 100 kilowatts was used. In 1915, at his suggestion, work on Directional Wireless was begun at the National Physical Laboratory. As Chairman of the Radio Research Board, his wise guidance and his enthusiasm for his subject have in no small measure contributed to the success of the important investigations on the fundamental problems of radio-transmission carried out under the auspices of that Board.

Secretion and the Golgi Apparatus in the Cells of the Islets of Langerhans.

By R. J. LUDFORD and W. CRAMER.

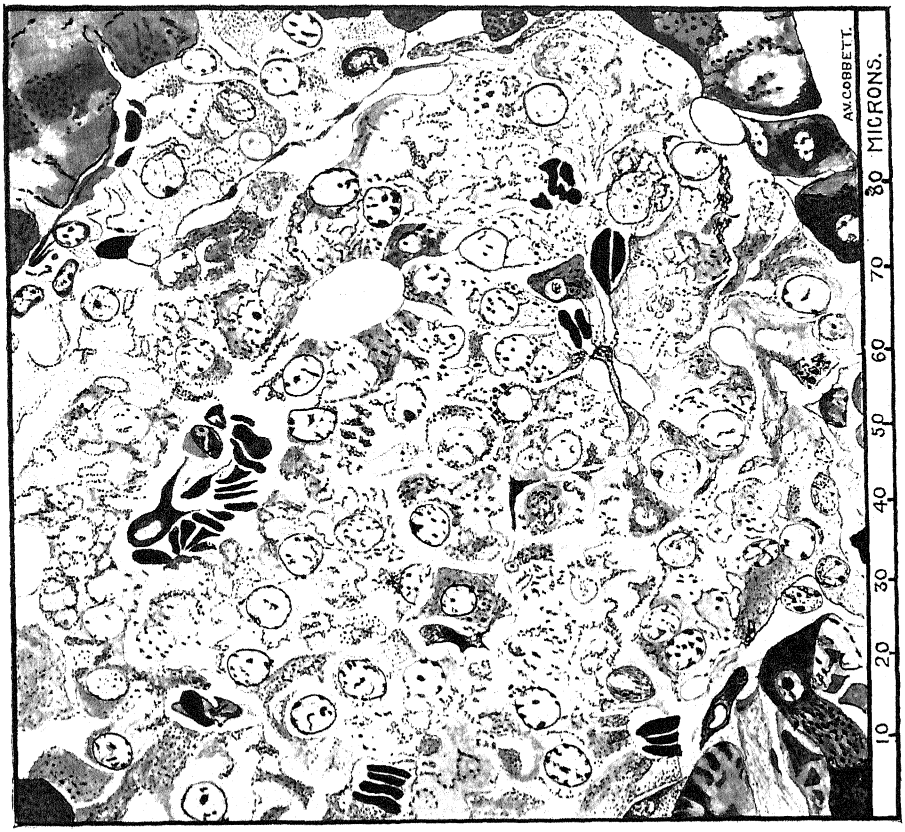
(Communicated by Dr. J. A. Murray, F.R.S.—Received October 20, 1926.)

(From the Laboratories of the Imperial Cancer Research Fund.)

[PLATE 1.]

Observations on the cells of the thyroid and of the adrenal medulla of animals placed under conditions which are known to stimulate the functional activity of these organs, have rendered it possible to correlate the functional activity of these endocrine cells with definite morphological changes, both as regards form and size of these cells, and their finer cytological structure, especially mitochondria and Golgi apparatus. Conversely, by excluding stimulating factors, it has been possible to produce a condition of rest in these endocrine organs in which the cells present a fairly uniform appearance. It is unnecessary to refer to these changes in detail, as they have been described and figured in several publications (Cramer, Cramer and Ludford). It is sufficient to make the general statement that in these two glands the functional activity of the cells manifests itself by morphological changes, which may be very considerable.

In the present investigations we have attempted to apply these considerations to a study of the islets of Langerhans. So far it has not been possible to obtain experimental evidence of the factors which control the secretory activity of the islets. From the beginning we meet here with the difficulty that the cells of the islets of animals, such as the rat, mouse and cat, on which our observations were made, present very marked differences in the size and arrangement of the cells, and also in their finer cytological structure, although the animals have been kept under apparently identical conditions. That is true not only if we compare the islets of animals of the same species, but even if we compare the islets in the pancreas of one and the same individual, or even the different cells in one and the same islet. Fig. 1 (Plate 1), for instance, which represents islets from two normal rats, fixed by Schridde's method and stained for mitochondria, shows great differences in the size and arrangement of the cells and in the size and number of mitochondria. These findings indicate that different islets of one individual, and even different cell groups of the same islet, are in


AV. COBBETT.
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80
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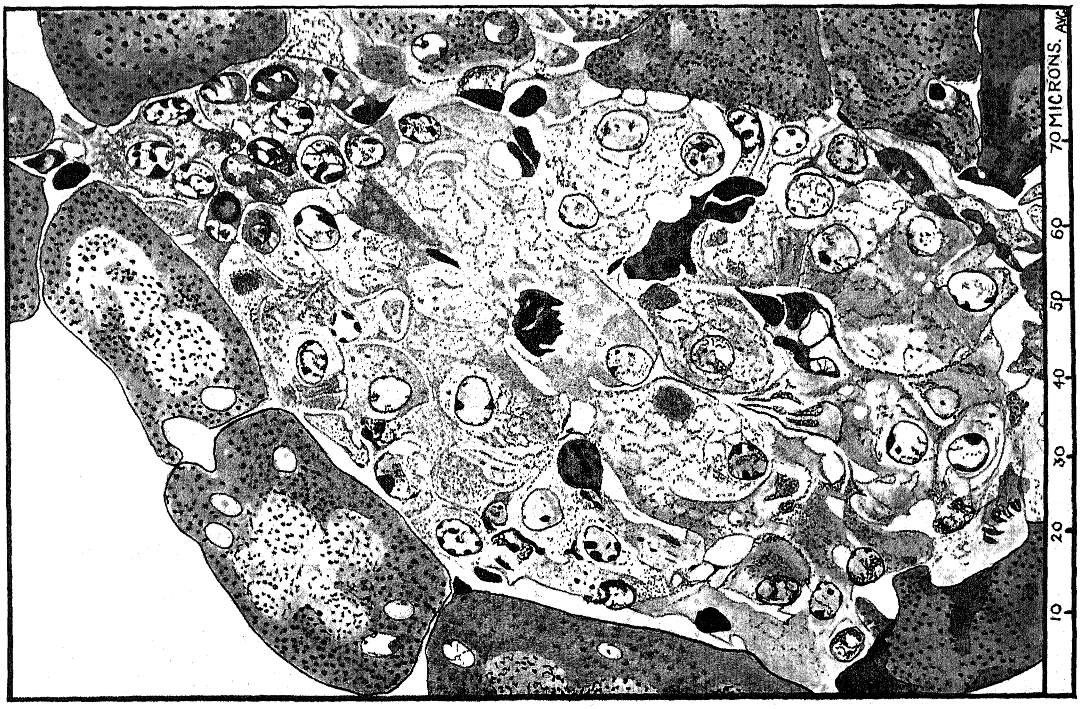
Fig. 1.


70 MICRONS. AVC
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different conditions of functional activity. This is confirmed by a study of the Golgi apparatus of the islet cells.

Recent investigations on the cytology of gland cells have established a close parallel between functional activity of gland cells and variations in the form of the Golgi apparatus. Several workers have described and figured differences in the form of the apparatus in various cells of the islet, and Bowen in his recent 'Studies on the Golgi Apparatus in Gland Cells' has suggested that the differences he found in islet cells of the cat's pancreas were due to different phases of secretory activity. We have observed the same phenomenon in the cells of islets of mice and rats. Fig. 2 shows the great variability in the form and arrangement of the Golgi apparatus in one islet from a normal mouse. It also shows the complete absence of polarity of the apparatus. Certain granules, marked *g*, of a fatty nature are conspicuous in some of the cells. Their significance is unknown.

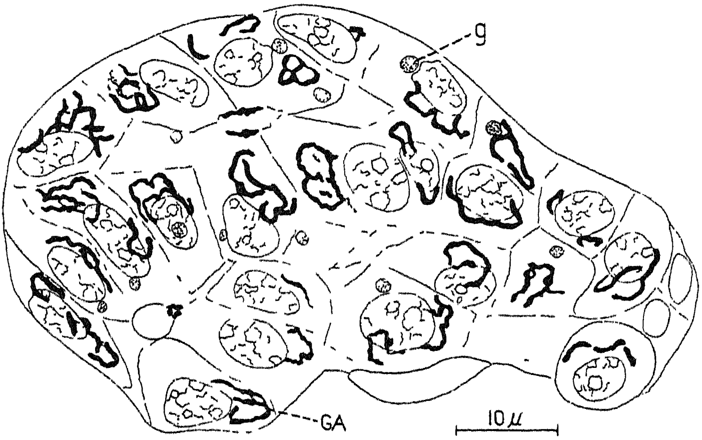


FIG. 2.—A complete islet from the pancreas of a normal mouse, showing the absence of polarity in the distribution of the Golgi apparatus (GA) and the presence of granules *g* in certain of the cells.

Lettering on all figures :—

GA = Golgi apparatus.
f = fragments impregnated by osmic acid, probably broken off from the Golgi apparatus.
g = granules, probably of a secretory nature, which may be derived from the Golgi apparatus.
s = secretion droplets or granules.

With the exception of fig. 1, all figures are drawn from preparations made by the modified Kopsch method for the demonstration of the Golgi apparatus.

A study of the Golgi apparatus confirms, therefore, the conclusion based on the other morphological characters of the islet cells, that in animals kept under the ordinary normal laboratory conditions the islet cells vary greatly in the state of their functional activity. We have attempted, therefore, to see whether, by varying these conditions, it is possible to obtain a greater uniformity of the islet cells and thus to obtain information on some of the factors which control the secretory activity of the islets of Langerhans.

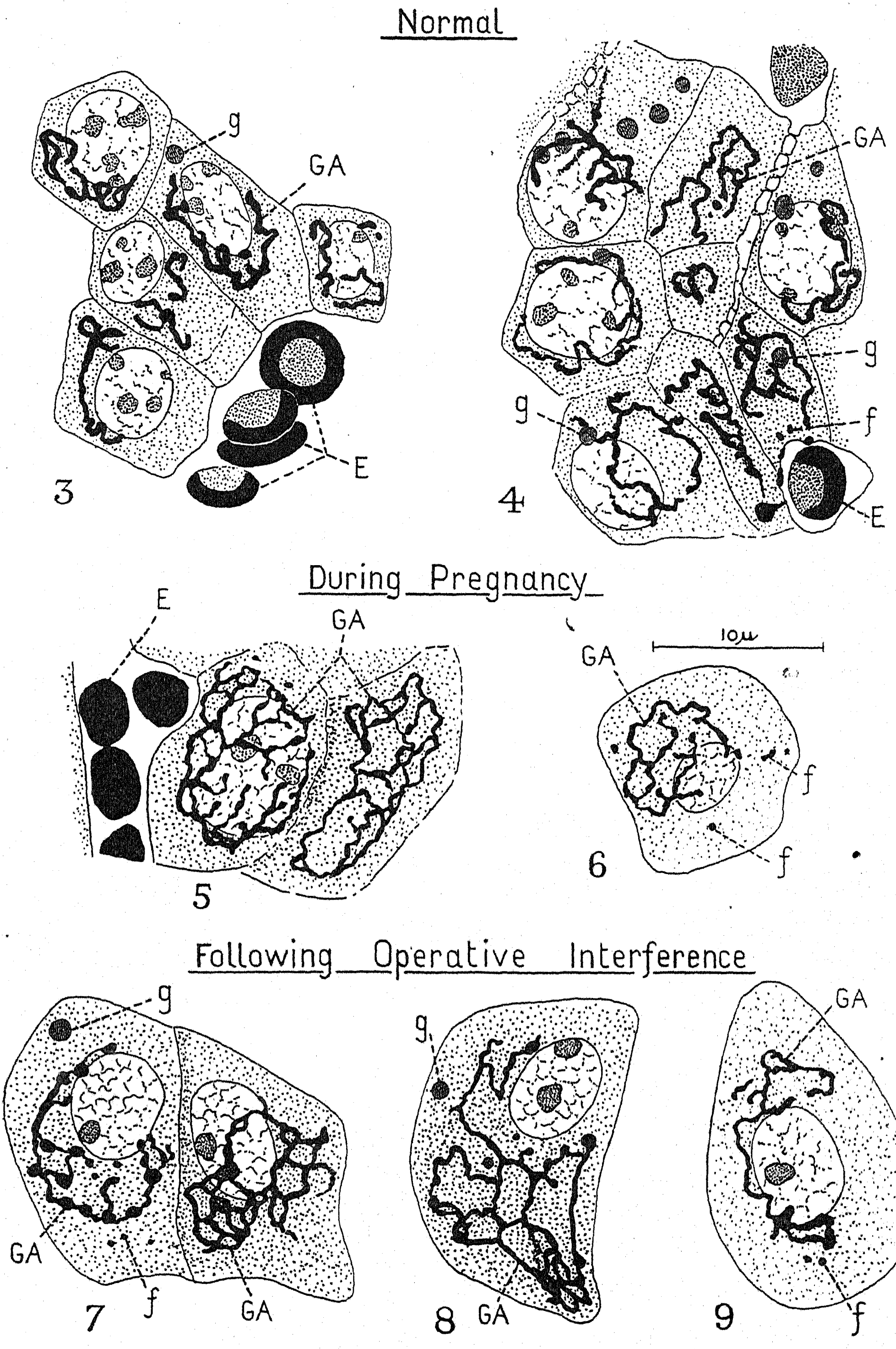
Rather striking cytological changes have been found to be associated with pregnancy and, in a less degree, to follow prolonged exposure to heat. It should be understood, however, that the changes described here apply only to a majority of cells comprising the islets, and not to the whole of the cells of any one islet. The different appearances which the Golgi apparatus may assume in different conditions are shown in figs. 3 to 9. These are cells from the mouse pancreas. Figs. 16 to 22 show similar changes in cells from the islets of the rat.

In addition to the variation in form of the Golgi apparatus, GA, shown in figs. 3 and 4, drawn from the normal mouse pancreas, attention is directed to the breaking away of osmophil fragments, *f*, from the apparatus, shown particularly in fig. 4. These granules are seen passing towards a capillary. It is of interest to note that similar osmophil granules have been seen associated with the apparatus in many gland cells during functional activity. We suggest that such granules represent a stage in the formation of the specific secretory products of the cell.

A great number of the cells of the islets of pregnant animals show a characteristic enlargement, and their Golgi apparatus, GA, also becomes considerably enlarged (figs. 5 and 6). Many of the cells show at times a coarse granulation of the cytoplasm. In some of the large cells the apparatus is broken up into fragments, *f*, as occurs after intense secretory activity in other gland cells.

Similar changes have been found in the animals in which the pancreas had been subjected to operative interference during splenectomy. In that operation a catgut ligature was placed around the tail of the pancreas. After the operation the animals had been kept in a specially warm room. Figs. 7 to 9 show some of the enlarged cells from the islets of such animals. Bead-like swellings occur on the reticulum of the apparatus in some of the cells. From the close parallel to the condition found in other gland cells, these swellings may be taken as the secretion forming inside the threads comprising the apparatus. Small osmophil fragments, *f*, to which reference has been made, also occur in most of these cells.

If sections which have been prepared for the demonstration of the apparatus



FIGS. 3–9.—Variations in the form of the Golgi apparatus in islet cells of the mouse, under varying conditions.

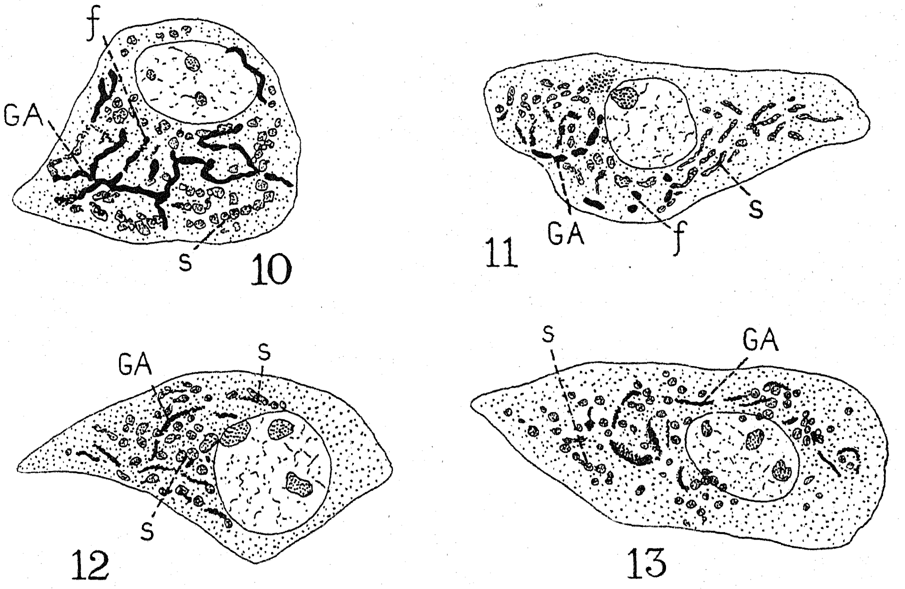
are partially bleached with potassium permanganate and sulphurous acid, and then stained with Altmann's aniline acid fuchsin, a number of granules can be seen in the cells of islets of animals kept in a hot room (figs. 10 to 13). A part of this granulation is undoubtedly mitochondrial, but not all of it. Certain of the granules, *s*, have a linear arrangement and appear to be continuous with the blackened part of the Golgi apparatus. The appearances suggest that in these enlarged cells secretion is being formed in association with the Golgi apparatus, and that this secretion passes out into the cytoplasm of the cell when the apparatus disintegrates. Very similar are the appearances seen in many of the cells of the islets during pregnancy.

Figs. 14 and 15 show cells from the islets of pregnant rats. In each of the cells the apparatus is broken up and granules or droplets occur in association with the broken fragments. The products of secretory activity seem to be passing out from the cells into a capillary (E, erythrocyte within capillary). It seems possible that droplets of secretion may be formed within the substance of the apparatus, or that osmophil fragments, *f*, may be broken off from the apparatus, and that these may become converted into the granules, *s*, which are then discharged as droplets of secretion.

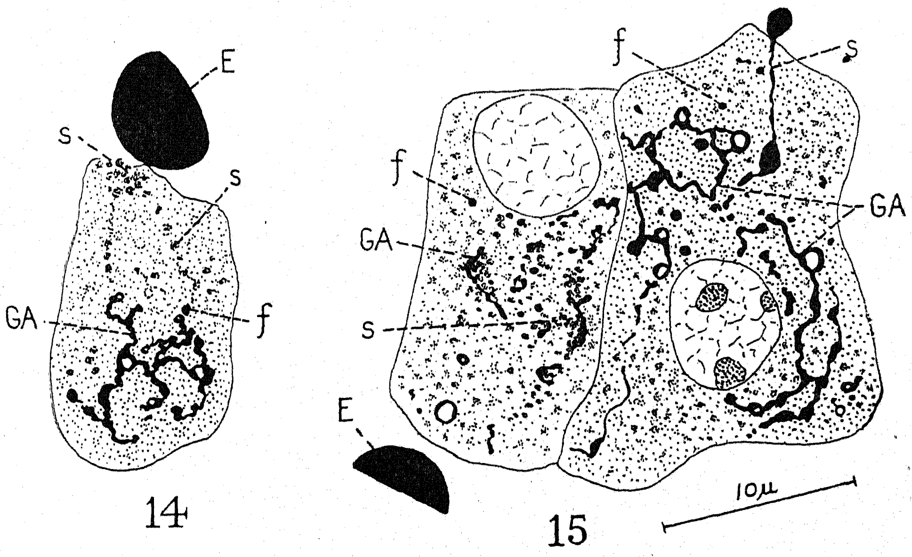
Figs. 16 to 22 show changes in the islet cells of the rat corresponding to those shown in figs. 3 to 9 in the case of the mouse. Figs. 16 to 19 indicate the wide range of variation in the apparatus of the islet of an animal kept in the cold. In comparison with the appearance of islets examined after exposure to heat, it may be concluded that the islet cells of animals kept in the cold are relatively inactive.

The enlargement of the cells during pregnancy is indicated in figs. 20 and 21. Fig. 21 is drawn under a lower magnification than the other figures. It indicates the dilatation and congestion of the capillaries of the islets occurring in pregnant animals and the somewhat acinar-like grouping of the cells around the capillary. *a*, *b*, *c* and *d* indicate what are regarded as successive stages in the form of the Golgi apparatus during secretory activity. A somewhat similar grouping of islet cells is shown in fig. 22, drawn from the islet of a rat which had been kept in a warm room. As in the previous figure *a*, *b*, *c* and *d* indicate the probable sequence of phases of cellular activity. This figure shows clearly the fragmentation of the apparatus and the discharge of the osmophil granules towards the capillary in the centre of the figure.

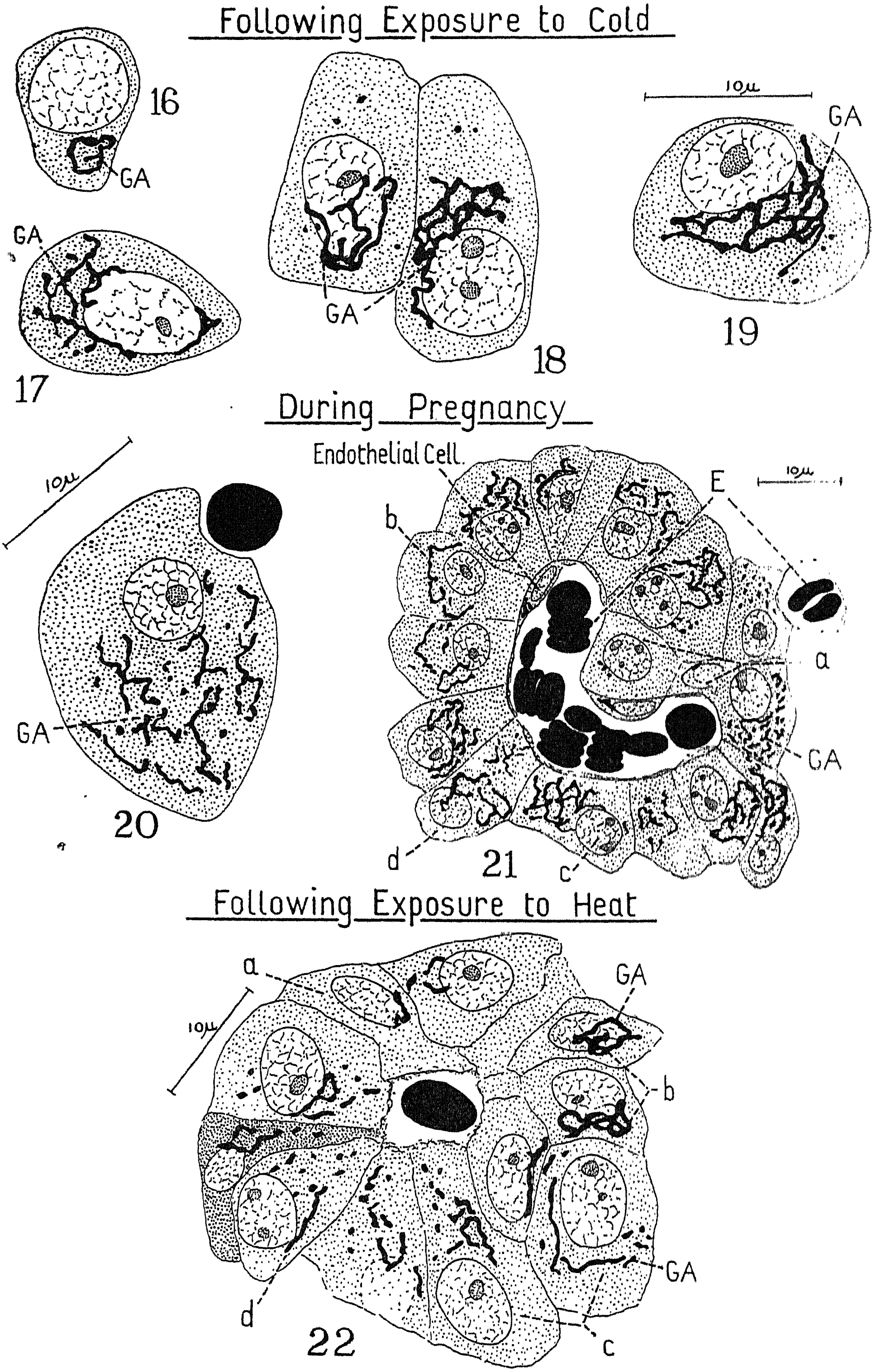
Following Exposure to Heat



During Pregnancy



Figs. 10–15.—The breaking up of the Golgi apparatus (GA), and the formation of products of secretory activity (*f*, *s*) in the cytoplasm. Figs. 10–13, cells from islets of mice kept at 37° C. for periods varying from 7 to 20 days. (Modified Kopsch preparation bleached and stained with aniline acid fuchsin.) Figs. 14 and 15, cells from islets of pregnant rats showing similar secretory products in the cytoplasm.



Figs. 16–22.—Variations in the form of the Golgi apparatus in islet cells of the rat, under varying conditions.

Conclusion.

The appearances in the cells of the islets of Langerhans in normal animals indicate that the cells of the islets in the same animals are at different stages of functional activity, and that this holds good even for the groups of cells in one and the same islet, so that at any one instant the various islet cells are exhibiting various phases of secretory activity. The condition in which the most definite and uniform change has been observed is during pregnancy, in which the islet cells are particularly active. A similar, but less uniform and less definite, change has been found after exposure to heat.

A pictographic schema of the secretory cycle in an islet cell is given in the accompanying figure (fig. 23).

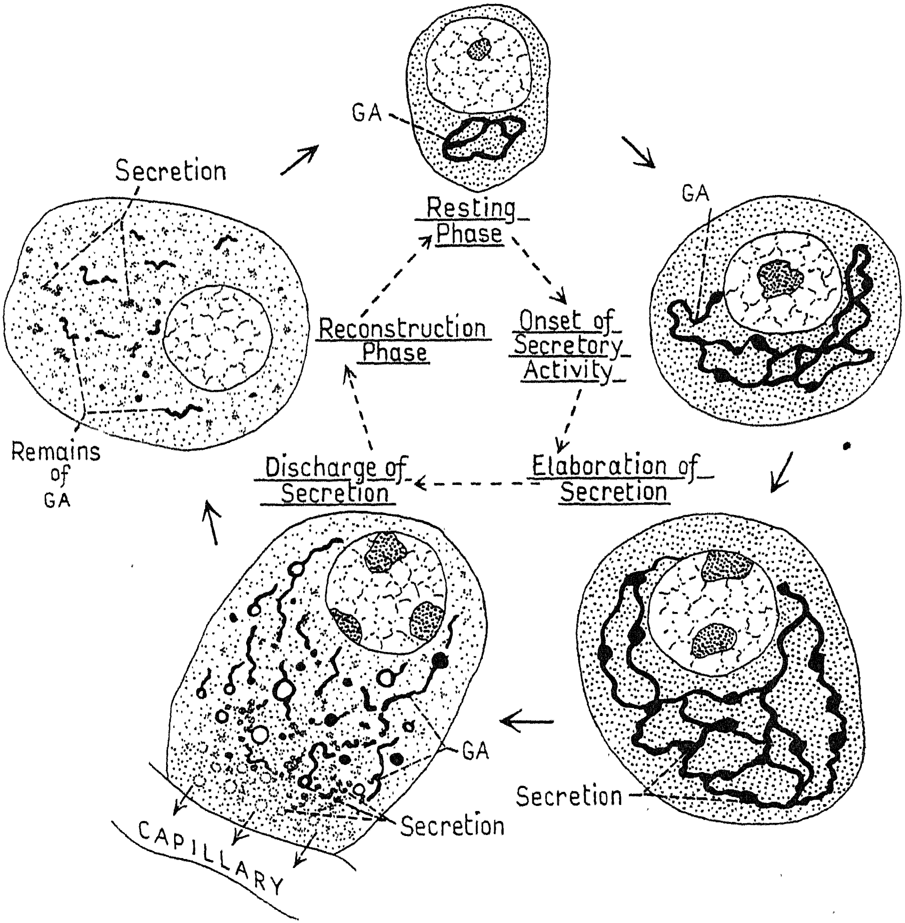


FIG. 23.—Pictographic summary of the secretory cycle in the islet cells.

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DESCRIPTION OF PLATE I (FIG. 1).

Two islets from two normal rats, showing the differences in size and arrangement of the cells. (Schridde method.)

Effects of Ultra-Violet Rays upon Skeletal Muscle.

By YOICHI AZUMA, Tokio, Japan.

(Communicated by Dr. L. Hill, F.R.S.—Received October 30, 1926.)

(From the National Institute for Medical Research.)

Leonard Hill and the author* have studied the contracture of involuntary muscle produced by radiation with ultra-violet rays, and the author† has shown that the presence of calcium ions in the nutritive solution is necessary for this contracture. The mechanism of the contracture produced by these rays is still undetermined. Leo Adler‡ considers it due to changes in the cells of the involuntary muscles, and not to excitation of the vegetative nerve system, which has so great an influence upon rhythmical movement as well as tonus.

On the suggestion of Dr. Leonard Hill the author investigated the action of the rays on skeletal muscle, but before doing so studied the effect of exposing various materials (cotton, linen, artificial silk and real silk-thread) to the mercury-vapour lamp. There might be a slight shortening of such fibres, but no remarkable difference in length occurred on irradiation. Tendon or skeletal muscle behaves quite differently from such dead fibres, showing distinct shortening on irradiation.

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* L. Hill and Y. Azuma, 'Roy. Soc. Proc.,' B, vol. 99, p. 221 (1926).

† Y. Azuma, "Effect of Ca and K-Ions on Involuntary Muscles," 'Roy. Soc. Proc.,' B, vol. 100, p. 431 (1926).

‡ L. Adler, 'Archiv f. Exper. Pathol. u. Pharmak.,' vol. 85, p. 153 (1920)

The method of experiment followed was nearly the same as that used in the case of involuntary muscle. Two mercury-vapour lamps (air-pressure type) were used, one placed on each side of the chamber in which the muscle was suspended. The chamber had a vitreosil plate on each side, through which the rays, including the ultra-violet rays from the M.V.L.'s, passed and acted on the muscle. Any heating effect during irradiation was avoided by irrigation with tap water, which ran down along the outside of either vitreosil plate. The nutritive solution used was Tyrode's and oxygen was bubbled through it. Bearing in mind the fact that the biologically active ultra-violet rays penetrate very little through living substance, the thin sartorius muscle of the frog was chosen and suspended with its breadth normal to the rays.

In the experiments on the action of different kinds of kations, the following was used as the fundamental solution, and the particular kations added to this: $NaCl$ 0·65, NaH_2PO_4 0·005, $NaHCO_3$ 0·1, H_2O 100.

I.

A frog's sartorius muscle, suspended in the nutritive solution, is in a quiescent state after a quarter of an hour. When exposed to the M.V.L.'s it begins to contract, in about a minute; the rate of contracture is at first very slow and then gets gradually quicker; it is most remarkable in about four minutes, and then again becomes slower; after ten minutes shortening is scarcely noticeable. If the exposure is then stopped, the contracted muscle begins to relax, and recovers nearly its normal length in 20 to 30 minutes. The relaxed muscle is, however, quite different from the fresh one, for when again exposed to the M.V.L.'s, it either does not contract, or if at all very slightly (fig. 1).

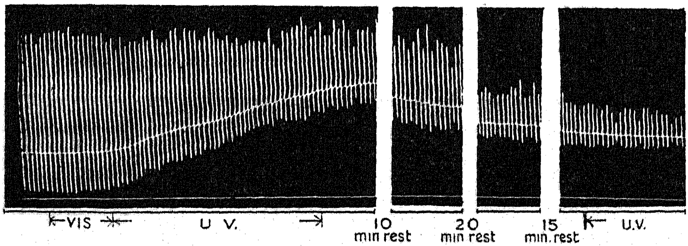

FIG. 1.

Involuntary muscle such as the frog's rectum does not lose its contractility on irradiation so quickly as the sartorius, a skeletal muscle, does. If the rays from the M.V.L.'s are screened by thick window glass to cut off the far ultra-violet rays, the contracture does not take place (fig. 1, vis). The effect of adrenalin in stopping the contracture of skeletal muscle was insignificant. A concentration was used which was enough to inhibit contracture of involuntary muscle (1 : 20 millions).

On stimulating the muscle with an electric shock every quarter of a minute while exposing it to the lamps, the amplitude of each twitch became smaller and smaller, until the muscle at last ceased to twitch (fig. 1). Increasing the strength of the stimulus might again provoke a twitch, but this soon ceased to be effectual. The excitability usually did not recover when the contracted muscle had relaxed again to its normal length.

Making a nerve-muscle preparation of the sartorius and exposing that to the ultra-rays, it was found that excitability of the nerve ceased before the direct excitability of the muscle. The nerve-endings appeared first to be paralysed. It seems probable that the contracture is due to a kind of "rigor mortis," and it would be of interest to examine the chemical changes of muscle after irradiation. Bearing in mind the small penetration of the active rays, a large number of sartorii of frogs would have to be used for this purpose.

II.

The effect of different kations (Ca, K and Mg) on the contracture was next investigated, just as had been done on involuntary muscle, and it was found that an adequate amount of calcium chloride had to be present in the nutritive solution in order to get contracture on irradiation, just as in the case with involuntary muscle.

If calcium ions are left out of the solution, there is no shortening, or, if any, only a very slight one, even if the muscle is exposed to ultra-violet rays for a long time. The moment the proper amount of calcium chloride is added the muscle starts suddenly and distinctly to contract (figs. 2, 3, 4 and 5).

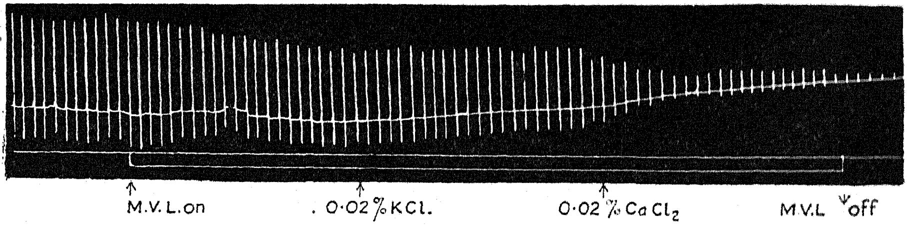

FIG. 2.

The contracture of the skeletal muscle exposed to ultra-violet rays in a nutritive solution, from which potassium and magnesium chloride are left out, but containing $CaCl_2$ (0·02–0·04 per cent.), is usually greater than that in the normal solution. That is to say, potassium and magnesium ions in the normal Tyrode solution moderate the action of calcium ions (fig. 4). The addition of calcium ions to the fundamental solution may temporarily increase the response to excitation (fig. 4).

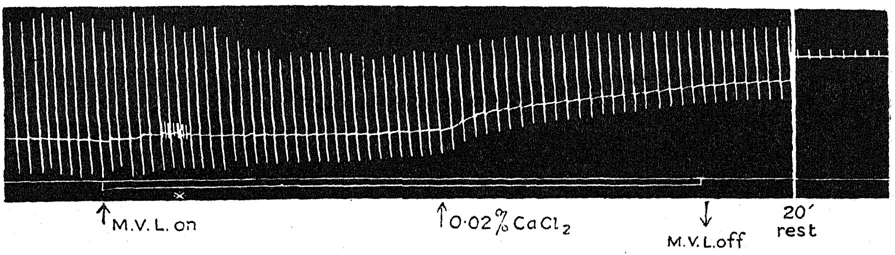

FIG. 3.

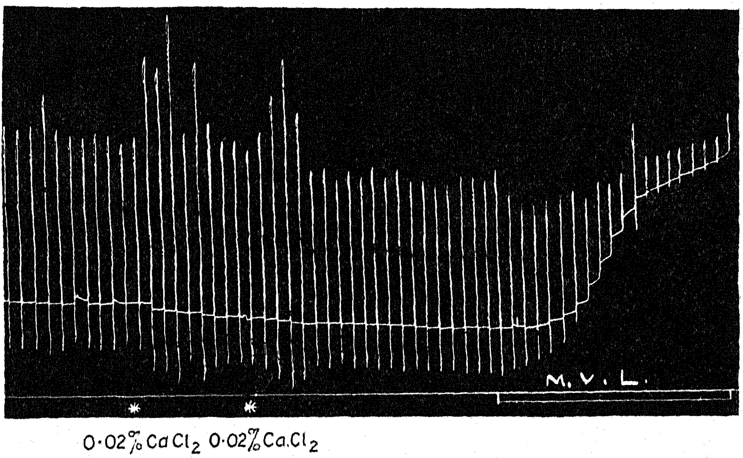

FIG. 4.

Relaxation of involuntary muscle has sometimes been brought about by means of magnesium chloride, acting contrary to calcium chloride; but this has not been observed in the case of skeletal muscle (fig. 5).

The addition of potassium chloride produced some increased contraction of the muscle on electric stimulation, but no shortening of the muscle on exposure to ultra-violet radiation (fig. 6).

Muscles other than the sartorius, for instance that of the frog's abdominal wall and the pectoral and tongue muscles, and similar muscles of the toad's

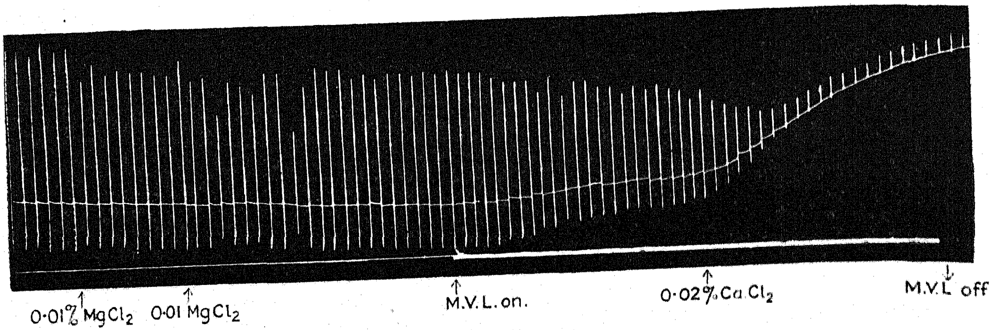

FIG. 5.

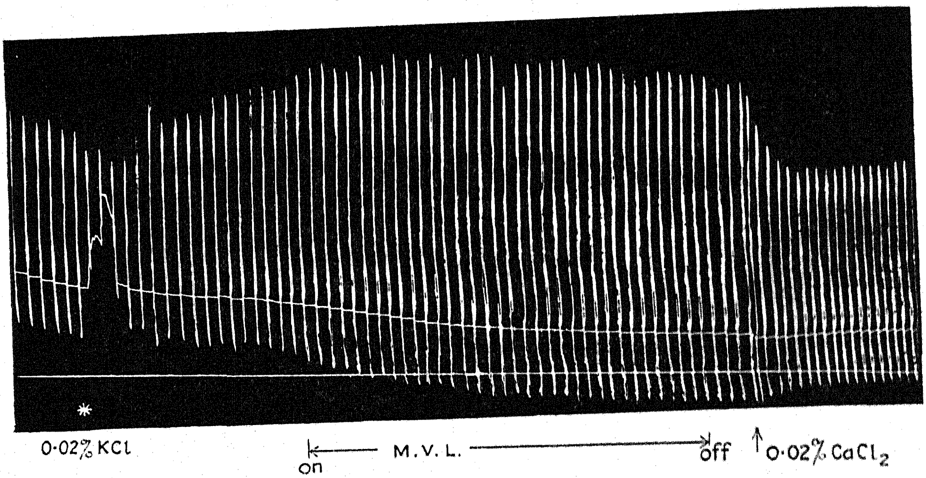

FIG. 6.

body, showed contracture on irradiation, but not to be compared with that of the frog's sartorius muscle, which is so thin and long that it is very suitable for exposure to the rays.

Conclusions.

(1) Skeletal muscle such as frog's sartorius suspended in Tyrode solution contracts remarkably on irradiation with ultra-violet rays.

(2) The contracture ceases after the irradiation and the muscle can recover its normal length, but the relaxed muscle cannot again be shortened by the ultra-violet rays.

(3) The excitability of the muscle to the electric shock becomes less and less when exposed to the ultra-violet rays and going into contracture. The excitability does not recover, although the contracture may pass away.

(4) The presence of calcium ions is necessary for the production of the con-

tracture by ultra-violet radiation. Potassium and magnesium ions seem to moderate the action of calcium ions.

(5) The contracture of the irradiated skeletal muscle is probably a kind of "rigor mortis," a "light rigor" which may be compared with "heat rigor."

The author expresses his gratitude to Dr. Leonard Hill for much help.

Changes in the Ovary of the Mouse following Exposure to X-Rays. Part I.—Irradiation at Three Weeks Old.

By F. W. Rogers Brambell, Fellow of the International Education Board; A. S. Parkes, Beit Memorial Research Fellow; and Una Fielding.

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(From the Department of Histology and Embryology, the Department of Physiology and Biochemistry, and the Department of Anatomy, University College, London.)

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I.—INTRODUCTION.

In a recent paper (Parkes, 8) the effect on the œstrous cycle of destruction of the Graafian follicles at three weeks old by X-rays was described, and the conclusion was reached that the absence of follicles does not inhibit the appearance of the œstrous cycle when the female subsequently becomes mature. In this earlier paper, however, the histological effects in the ovary after exposure to X-rays were only dealt with very briefly, and it is the object of this present paper to consider in detail the histological changes following irradiation at weaning time. The histological effects of irradiation at birth and of irradiation of the adult, together with the effects on the œstrous cycle, will be dealt with in subsequent communications. Two main problems are presented by the histology of these irradiated ovaries. In the first place, the exact fate of the disintegrating follicle is of considerable importance. The maturation of follicles has long been considered to be essential for the appearance of œstrus, and evidence has recently been adduced to show that the hormone responsible for œstrus is elaborated by the follicle. Various elements of the follicle have been held to be responsible by various authors. Allen and Doisy (1), for instance, suggest the follicular epithelium, while Zondek and Aschheim (16) maintain that the theca interna is concerned. Since, therefore, the irradiated ovaries are capable of elaborating œstrin* it is essential to know whether the disappearance of follicular tissue is complete or only partial. As reported previously, it is possible to say with certainty that ovum and follicular epithelium disappear entirely, except where the latter helps to form corpora lutea atretica, but the fate of the theca interna is less obvious. This latter question is, however, of importance from a physiological point of view.

The second main problem connected with these ovaries is the nature of the cords of the second post-irradiation proliferation which ultimately arise in most of the ovaries after irradiation, and which were tentatively suggested in the earlier paper to be of spermatic character.

Amongst earlier workers there is considerable agreement concerning the complete degeneration of the follicles following X-ray irradiation. Regaud and Lacassagne (11) give the fullest account. They describe the ovaries of eight rabbits. They find that the primary follicles exhibit degeneration of the oocyte in from 15 hours to 4 days according to the strength of the dose. They believe that those which persist for one week have definitely escaped. The follicle cells take on a phagocytic function and absorb the oocyte. Later they themselves degenerate and are absorbed, and usually have completely disappeared by the end of the first week. In follicles with two to four layers of cells the degeneration of the oocyte is followed by the penetration into it of follicular epithelial cells, the nuclei of which remain separate, though the cytoplasm fuses. These giant multinucleate cells lie in cavities in the stroma and gradually autolyse in 10 to 15 days. In many layered follicles the nucleus of the ovum sometimes divides and the cytoplasm segments. The follicle cells degenerate more rapidly than in earlier follicles and the

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* Suggested elsewhere (Parkes and Bellerby, 9) as a convenient term for the œstrus-producing hormone.

follicles disappear in two to four weeks. In mature follicles the nuclei always exhibit degenerative polar-body formation and segmentation. The follicular epithelium is entirely killed by the X-rays and does not take part in the destruction of the ovum. The theca cells are also destroyed and removed by polymorph leucocytes. In some cases intra-follicular hæmorrhage results in cyst-formation. The interstitial tissue begins to atrophy after three to four weeks; they believe owing to the destruction of the normal source—the theca interna cells of atretic follicles. After three months, new interstitial tissue is found to be forming from cortical connective tissue cells. The new interstitial tissue is formed slowly at the periphery at the same time as the old interstitial tissue is degenerating in the centre. It seems clear that the new interstitial tissue of Regaud and Lacassagne corresponds to our first proliferation. These authors (10) also describe as anovular follicles structures obviously similar to our cords of the second proliferation, but in a later paper (12) they come to the conclusion that they are constituents of the normal ovary and not due to the X-irradiation.

Bouin, Ancel and Villemin (3 and 4) maintain that in the X-rayed rabbit, after the disappearance of the follicles, the interstitial gland constitutes almost the whole of the ovary and preserves its morphological integrity.

Bergonié and Tribondeau (2), working on the rabbit also, come to exactly the opposite conclusions. They find that the total volume of the interstitial tissue is diminished, the nodules composing it are more widely separated and the individual cells become shrunken and their nuclei angular. They believe that it disappears owing to the destruction of its normal source, the theca interna cells, and not owing to the direct effects of the X-rays.

Steinach and Holzknecht (13), in an X-rayed virgin guinea-pig two to four weeks old, find that all the follicles are completely atrophied and the contents shrivelled up to form necrotic masses or hyaline lumps. The whole ovarian stroma was an almost unbroken mass of female "puberty-gland." It shows lutein-cell characters and is in part rich in fat-granules. They do not mention the source of this mass, which apparently corresponds to our first proliferation.

Reference should be made to the above-mentioned papers for a full bibliography.

II. DESCRIPTION OF CASES.

Material.—The full data of the œstrous cycles of the majority of the animals described in this paper are recorded in a previous paper by one of us (A.S.P. (8)). In the present paper the data of M 42, 49, 50, 51 and 57 are given in full, as they were not included in the previous paper. M 15 and 16 will be described more completely in a later paper, as only the first ovary removed comes within the scope of the present one. Vaginal smearing was continued for some time after double ovariotomy in M 11, 13, 17, 20, 21, 22, 23, 25 and 27, and in all the œstrous cycle was found to have stopped completely, showing that the ovaries removed were responsible for its production. In all cases the time of death or ovariotomy is calculated from the first irradiation.

The technique of the dosage and the intensity of the irradiation have been described elsewhere. The early animals, which were not uniformly sterilised, were given an irradiation of 40 minutes, corresponding to roughly ⅛ B tint on Levi's pastilles. The later animals (6-27, 42, 49-51, 57) were given double this dosage, and were uniformly devoid of Graafian follicles. Subsequently other animals (36-41) were given the same dosage and killed at weekly intervals for the purpose of ascertaining the immediate post-irradiation changes in the ovary.

The material was fixed in Bouin's or Carnoy's fluid and stained in Ehrlich's hæmatoxylin and eosin, or in Haidenheim's iron hæmatoxylin. Champy's fluid was also used, followed by iron hæmatoxylin or the Champy-Kull method. Complete serial sections were made of the ovaries.

The Series.—M 36 was killed 7 days after the first irradiation. The oocytes present are all more or less full size, but in follicles of varying size. Many of the oocytes show signs of breaking down and exhibit degenerative cleavage and segmentation (Plate 3, fig. 3). In some of the follicles the membrana granulosa is breaking down also. Some follicles are only represented by the crumpled and shrunken oocyte surrounded by a few necrotic epithelial cells within the thin sheath of thecal fibroblasts. No corpora lutea atretica are present. The stromal tissue appears slightly hypertrophied in places, and no epithelial proliferation is taking place. The tunica albuginea is well developed.

M 37 was killed 14 days after irradiation. The oocytes are all full size, and they and the follicles show various stages of degeneration. Some of the follicles are small, others large and with a fairly developed antrum. The most degenerate are only represented by a thin-walled cavity containing the shrunken eosinophil zona pellucida. No corpora lutea atretica are present. The old stromal tissue between the follicles is loose and unhealthy looking. The tunica albuginea is well developed and the germinal epithelium is thickened and beginning to proliferate a few cords.

M 38 was killed 21 days after irradiation. No normal oocytes or follicles are present. Cavities with zona pellucida remnants are present in the central regions. In the larger follicles in which it is differentiated the theca interna is breaking down. In these the membrana granulosa degenerates before the theca interna. No corpora lutea atretica are present. The old stromal tissue cells are necrotic, vacuolated and fat-laden, and appear unhealthy. The germinal epithelium has started proliferating, a few small cords being present in the periphery. The tunica albuginea is not well developed, but is intact, except where the epithelial cords, to which it has contributed sheaths of fibroblastic cells, have penetrated it.

M 39 was killed 28 days after irradiation. All the oocytes and follicles are degenerating or becoming atretic. Cavities with zona pellucida remnants are present and in some cases the cells of their walls appear to be growing and forming a ring of epithelioid cells around and outside the fibrous wall. These cells are not like theca interna elements, none of which appear to be present. This condition was seen also in M 1*a*. Several of the larger follicles are forming corpora lutea atretica, but the antrum is not completely filled. The germinal epithelium is not much thickened, but is actively proliferating cords which penetrate and disturb the tunica albuginea. The cords occupy the spaces between the old follicles in the periphery. Several are so well formed that they almost resemble those of the second proliferation.

M 40 was killed 35 days after irradiation. It is very similar to the previous case. Several corpora lutea atretica are present, only a small central cavity in them representing the antrum of the follicle. All the other follicles are represented by small cavities, containing zona pellucida remnants, which have been pushed into the centre by the growth of the epithelial cords of the first proliferation, the cells of which have enlarged somewhat and tend to become a little luteal-like. There appear to be a few cords of the second proliferation.

M 41 was killed 42 days after irradiation. The ovaries are similar to M 40, except that one contains a small apparently normal follicle and the other two abnormally large ones, the antrum of each of which is filled with blood. This case is not, therefore, completely sterilised.

Adult animals.—M 19 was killed 39 days after irradiation. The ovary contains many cavities enclosing zona pellucida remnants. Some old stromal tissue appears to be present. The germinal epithelium is much thickened and is actively proliferating cords. The tunica albuginea is not well developed.

M 2*b* was killed 53 days after irradiation. Many cavities with zona pellucida remnants are present. The germinal epithelium is thickened and darkly staining. It is actively proliferating cords in the form of stout, solid finger-shaped processes extending down between the cavities which represent the old follicles. The tunica albuginea is well marked and trabeculæ of fibroblasts and connective tissue are growing, or being carried, down from it between the ingrowing cords.

M 2*a* was killed 53 days after irradiation. The ovaries are very similar to 2*b*, but the cavities are pushed in from the periphery by the growth of the cords and are in the centre. A few cords of the second proliferation are present in the periphery and can be seen in tangential sections pushing through the tunica albuginea.

M 1*b* was killed 53 days after irradiation during the only œstrous period observed. It is very similar to 2*a* and *b* and intermediate between them.

M 1*a* was killed 53 days after irradiation. It is similar to M 1*b*, but zones of cells, staining slightly more deeply than the tissue around them, surround the thecal capsules of some of the cavities. The appearance is the same as that of M 39. These cells may be membrana granulosa cells which have persisted. In this case the wall of the cavity they surround must have been formed within the membrana granulosa by fibroblasts which passed into the follicle from the outside.

M 3*a* was killed 53 days after irradiation. Epithelial cords of the first and second proliferation are present, and cavities with zona pellucida remnants occur. Follicle cells appear to be persistent round these. Two cysts occur with thin fibrous capsules and epithelial linings. The larger is about half the diameter of the ovary and both of them are filled with blood.

M 2*c* was killed 63 days after irradiation. Some cavities occur in the central region, where the tissue appears to be old stroma. Epithelial cords are growing in around this area. (Plate 4, fig. 3.)

M 1*c* was fertile and produced a litter 90 days after irradiation, when it was killed. The ovaries are normal, except that some cords of the first proliferation appear to be present in the periphery.

M 1*d* was killed 99 days after irradiation and two days after the fourth œstrous period observed. The ovaries are practically identical with those of M 2*a*. Plate 3, fig. 2, shows in plan the cords of the second proliferation penetrating the tunica albuginea.

M 2*d* was killed 87 days after irradiation, during the fourth œstrous period observed. One small cyst, lined with cubical epithelium, is present. A retrogressing corpus luteum atreticum is present in the other ovary and the ingrowing epithelial cells seem to be invading it. Cavities with zona pellucida remnants are numerous. The tissues are almost entirely composed of cord of the first proliferation. The germinal epithelium is thickened and deeply staining. Small cords of the second proliferation are found, one or two deep, around the periphery. The tunica albuginea is well developed.

M 3*b* was killed 88 days after irradiation and immediately after the second œstrous period observed. Cavities, the remains of old follicles, are present. One ovary contains a small corpus luteum atreticum. The tissue of the first proliferation is somewhat luteal-like. The germinal epithelium is thickened in places and a few cords of the second proliferation are scattered around the periphery. The tunica albuginea is well developed.

M 6*a* was killed 105 days after irradiation and three days after the second œstrous period observed. The cords of the first proliferation are in the form of thickened lobes projecting from the periphery in towards the loose medullary region. The cells have become large and rather luteal-like. The germinal epithelium is thin and inactive. The tunica albuginea is very thin but distinct. A few cords of the second proliferation, intermediate in type between those of the first proliferation and those formed later, are present in the periphery.

M 6*b* was killed 97 days after irradiation during the second œstrous period observed. A few cavities representing old follicles are present. One ovary contains a fairly large cyst in the hilar region, and the other what appears to be a corpus luteum atreticum. The tissue of the first proliferation is similar to that in M 6*a*. A few cords of the second proliferation are present in the periphery.

M 6*c* was killed 78 days after irradiation during the fourth œstrous period observed. A few cavities containing zona pellucida remains are present, but no corpora lutea atretica. The tissue of the first proliferation is not luteal-like. The germinal epithelium is actively forming cords of the second proliferation and both stain deeply, forming a very distinct zone around the periphery of the ovary (Plate 4, fig. 1).

M 6*e* was killed 134 days after irradiation, no œstrous period having been observed. This animal, in many ways the most remarkable of the series, must be described at greater length. The first ovary was fixed in Bouin's fluid and stained with Ehrlich's hæmatoxylin and eosin. The medullary zone is loose and very vascular. Around it are the remnants of the tissue of the first proliferation. Almost the entire cortex is filled with "tubules" of the second proliferation (Plate 2, fig. 2). These are sub-equal in size, but are, on an average, larger than those usually found. They are very well developed, with a clearly marked fibrous sheath, a cubical lining epithelium and a well-marked lumen, in some cases clear, in others containing a few detached epithelial elements (Plate 3, fig. 1). These, therefore, in section very closely resemble spermatic tubules without spermatocytes. None of the nuclei, however, resemble in any distinctive character those of normal germ cells. The cells, however, appear healthy and are suggestive of Sertoli cells. Throughout most of the cortex these "tubules" are closely packed together. The greater part of the epithelium is thickened and actively proliferating. The tunica albuginea is present and fairly well developed. The "tubules" are never long and anastomosing, but in many cases are apparently nearly spherical, in others at least three or four times as long as broad. The cells of the first proliferation appear crushed into the centre by the pressure of the ingrowing cords of the second proliferation, in the same way as the medullary cords are compressed by the cortical in the embryonic ovary (as described by de Winiwarter and Sainmont (15) in the cat). The cells of this first proliferation show crenated nuclei and a large cytoplasm full of small vacuoles. They are still in the form of cords, but the fibrous sheaths of these, as in the other animals, are not nearly as well developed as those around the second proliferation of "tubules," which are very distinct. Absolutely no traces of old follicles, oocytes or corpora lutea can be made out in either ovary. The second ovary was prepared by the Champy-Kull method of fixation and staining. This ovary is similar to the other, but the cortex is not quite so filled with tubules of the second proliferation. There is a certain amount of osmicated fat to be seen in the cells of the first proliferation, and the method reveals the presence of several cavities containing the remnants of zona pellucida that were not observable in the other ovary. The germinal epithelium, which is almost columnar, is proliferating very actively and small groups of proliferated cells can be seen in it and immediately beneath it. There is every reason to believe that these grow into the tubules after they have passed in from the epithelium. The cells in the tubules vary in the extent to which they take the fuchsin, just as do those of a testis tubule. The cells of the lining epithelium have some fat globules at the poles next the lumen and the detached cells in the lumen have more scattered globules in them. In one or two cases some epithelial cells, more or less degenerate, are still present around the remnants of an old zona pellucida in a cavity representing an old follicle.

M 6*f* was killed 93 days after irradiation and two days after the third œstrous period observed. A few cavities containing zona pellucida remnants are present near the centre. The tissue of the first proliferation is luteal-like. The germinal epithelium is inactive and the tunica albuginea is very poorly developed. Only a very few cords of the second proliferation are present.

M 7*a* was killed 136 days after irradiation and 57 days after the only œstrous period observed. One ovary contains a corpus luteum atreticum. The cells of the first proliferation are large, very vacuolated and luteal-like (Plate 5, fig. 2). There is no second proliferation. Plate 4, fig. 2, shows in plan the cords of the first proliferation penetrating the tunica albuginea.

M 7*c* was killed 136 days after irradiation and 74 days after the only œstrous period observed. No corpora lutea atretica are present, otherwise the ovaries are exactly similar to M 7*a*.

M 8 was killed 66 days after irradiation and 9 days after the second œstrous period observed. Cavities with remnants of zonæ pellucidæ are present in the central region and two or three small corpora lutea atretica. The cords of the first proliferation form the major part. Cords of the second proliferation, intermediate in type between these and those formed later, are forming rapidly from the epithelium, especially in the region of the hilus. Some of the nuclei in these are round and full and sometimes exhibit mitotic stages, others are crenated and small, though apparently healthy. These two kinds are suggestive of the spermatogonia and Sertoli cells of spermatic tubules.

M 9 was killed 66 days after irradiation and 9 days after the second œstrous period observed. The ovaries are similar to those of M 8, except that no corpora lutea atretica are present.

M 10 was killed 66 days after irradiation and one day after the third œstrous period observed. A few cavities representing old follicles are present in the centre. The entire ovaries are composed of tissue of the first proliferation, no cords of the second being present.

M 11 was ovariotomised 94 days after irradiation and 27 days after the only œstrous period observed. The ovaries are exactly similar to M 10.

M 12 was killed 53 days after irradiation during the second œstrous period observed. Cavities representing former follicles are present in the central area and one small corpus luteum atreticum, with a large central cavity, is present in one ovary. The ovaries are chiefly composed of tissue of the first proliferation. There are many cords of the second proliferation, intermediate in type like those described in M 8. Some mitotic stages in these strikingly resemble spermatogonia divisions.

M 13 was ovariotomised 87 days after irradiation, during the second œstrous period observed. The ovaries are similar to M 12, but no corpora lutea atretica are present.

M 16 had one ovary removed 98 days after irradiation. There are no traces of old follicles or of corpora lutea. This ovary consists of a central mass of somewhat luteal-like tissue of the first proliferation with numerous cords of the second proliferation forming a broad peripheral band around it (Plate 2, fig. 4). The germinal epithelium is actively proliferating. The cords of the second proliferation exhibit many mitoses. They are better formed and more numerous than in any case except M 6*e*. Several long canals penetrate deep into the tissues of the ovary (Plate 5, fig. 1). They are narrow and uniform in diameter and the clear lumen opens on the surface. The cubical epithelium lining them is continuous with the germinal epithelium, and their fibrous sheaths with the tunica albuginea. They appear to arise as invaginations of the germinal epithelium, which carry in a sheath of the tunica albuginea with them.

M 15 had one ovary removed 98 days after irradiation. This ovary is similar to that of M 16, but the cords of the second proliferation are not so well developed and are more intermediate in type and the epithelial canals are not so long.

M 17 was ovariotomised 101 days after irradiation and one day after the fourth œstrous period observed. A few cavities representing old follicles are present. The tissue of the first proliferation is somewhat luteal-like and the cells contain much fat. Cords of the second proliferation form a peripheral band around the ovary.

M 18 was killed 89 days after irradiation and one day after the third œstrous period observed. There are no traces of follicles in the ovary, which almost entirely consists of tissue of the first proliferation. The germinal epithelium is thickened and the tunica albuginea is very well developed. The few cords present, representing the beginning of the second proliferation, are intermediate in character.

M 20 was ovariotomised 94 days after irradiation, and 11 days after the second œstrous period observed. There are no traces of follicles in either of the ovaries, both of which are chiefly composed of blunt finger-shaped cords of the first proliferation converging from the periphery on the loose medullary zone (Plate 2, fig. 1). The cells of these cords are large and luteal-like. The germinal epithelium is thickened and active, and the tunica albuginea is very thick and well developed. Cords of the second proliferation, intermediate in type, are present all around the periphery, but especially in the hilar region.

M 21 was ovariotomised 87 days after irradiation and four days after the second œstrous period observed. A cystic follicle is present in one ovary, but there are no other traces of follicles. The ovaries consist of somewhat luteal-like tissue of the first proliferation. The germinal epithelium is thin and inactive and the tunica albuginea is thin and poorly developed. There are no cords of the second proliferation.

M 22 was ovariotomised 71 days after irradiation and five days after the third œstrous period observed. There are no traces of follicles in the ovaries, which are chiefly composed of tissue of the first proliferation. The germinal epithelium is forming a few cords of the second proliferation, intermediate in type. The tunica albuginea is well developed.

M 23 was ovariotomised 78 days after irradiation and 12 days after the second œstrous period observed. The ovaries are similar to those of M 22.

M 25 was ovariotomised 78 days after irradiation and one day after the fourth œstrous period observed. A few cavities with zona pellucida remnants are present. The ovaries are composed entirely of somewhat luteal-like tissue of the first proliferation. The germinal epithelium is thin and inactive and the tunica albuginea is fairly developed. No cords of the second proliferation are present. Slight invaginations of the germinal epithelium suggest the beginning of epithelial canals similar to those described in M 15, 16 and 42.

M 26 was killed 69 days after irradiation and eight days after the third œstrous period observed. One old corpus luteum atreticum is present, but there are no traces of follicles. It is composed chiefly of cords of the first proliferation. The germinal epithelium is thickened and has formed some cords of the second proliferation, intermediate in type. The tunica albuginea is thick.

M 27 was ovariotomised 94 days after irradiation and 37 days after the only œstrous period observed. A few cavities with zona pellucida remnants are present. The ovaries are composed entirely of cords of the first proliferation, and none of the second are present. The germinal epithelium is thin and inactive and the tunica albuginea is well developed.

M 42 was put on vaginal smearing 52 days after the first irradiation, and examination was continued for 145 days. During this time 14 periods of œstrus, embracing 13 complete cycles, were observed, and nine of these were accompanied by copulation. The lengths of the cycles were:—14–14–11–14–12–7–6–8–9–9–7–10–7 days. Of these, the 1st, 2nd, 3rd, 5th, 6th, 7th, 10th, 11th and 13th were preceded by copulation. The œstrous periods observed were quite normal as regards the contents of the vaginal smears, cornification being observed each time. It was killed 195 days after irradiation and immediately after the 14th œstrous period observed. One large corpus luteum atreticum is present. The tissue of the first proliferation is rather luteal-like. The germinal epithelium is thin and almost inactive and the tunica albuginea is well developed. Short epithelial canals, similar to those described in M 15, and a very few cords of the second proliferation are present.

M 49 was put on vaginal smearing 69 days after irradiation, and was observed for 37 days. During this time only two periods of œstrus, 25 days apart, were observed. Each was attended by copulation. It was killed 102 days after irradiation and six days after the second œstrous period observed. One ovary contains a cyst, about half the size of a mature follicle, lined with cubical epithelium. A few cavities with zona pellucida remnants are present. Both ovaries are chiefly composed of the cords of the first proliferation, but there are also cords of the second proliferation, forming a well-marked peripheral zone. The germinal epithelium is thickened and actively proliferating and the tunicà albuginea is thick and well developed.

M 50 had an œstrous cycle history almost identical with that of M 49. It was observed for the same time. The two œstrous periods, both attended by copulation, were 22 days apart. It was killed 102 days after irradiation and 11 days after the second œstrous period observed. Some small cavities containing a pink-staining coagulum are present and probably represent former follicles. Both ovaries are chiefly composed of the cords of the first proliferation. The cords of the second proliferation are well developed and form a well-marked peripheral zone (Plate 2, fig. 3). The germinal epithelium and tunica albuginea are similar to those of M 49.

M 51 was also left 52 days after irradiation before examinations were begun. It was observed for 37 days, during which time four periods of œstrus, all attended by copulation, were observed. The three cycles were respectively of 8–4–13 days duration. It was killed 102 days after irradiation and 11 days after the fourth œstrous period observed. Two epithelial lined cysts, one large and one small, are present in the hilar region of one ovary, and a blood-filled cyst about the size of a mature follicle is present in that of the other. The ovaries are otherwise similar to those of M 49, except that no traces of follicles can be distinguished with certainty.

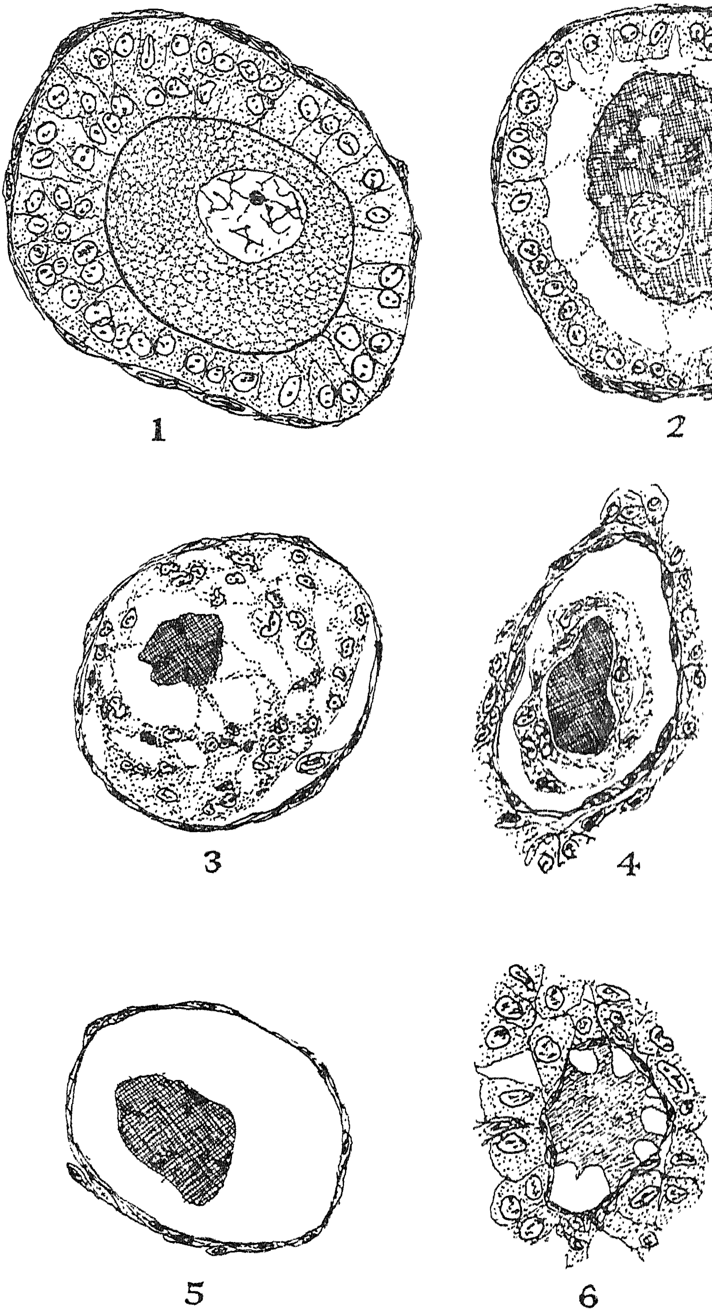
M 56 was killed 76 days after irradiation. No traces of follicles are present. Both ovaries are composed of small-celled tissue of the first proliferation which is not luteal-like, and contains fibroblasts. The germinal epithelium is thin and the tunica albuginea is well developed, with, in many places, small spaces between it and the underlying tissues. A few cords of the second proliferation, intermediate in type, are present, especially in the region of the hilus.

M 57 was observed for 20 days only, at the end of which time it became ill and had to be killed. During the time under observation two periods of œstrus (five days apart) were observed, the latter one only being accompanied by copulation. It was killed 93 days after irradiation and seven days after the second œstrous period observed. The ovaries are similar to those of M 50.

III. General Histological Description.

(a) Histological Changes following Irradiation.

The Follicles.—One week after the first dose of X-rays all the small oocytes have disappeared from both ovaries of the mouse, as shown by M 36 (Plate 3, fig. 3). Some of the larger oocytes, which were surrounded by a follicle, are only represented at this stage by a few shrunken remnants within a small cavity surrounded by a thin fibrous sheath. Those oocytes that persist, either normally or in early stages of degeneration, have all completed the growth stage and are full size. The first stages of degeneration of the oocytes approaching maturity are often represented by the formation of the polar bodies and segmentation, followed by complete fragmentation. Similar phenomena have been described in degenerating ovarian and uterine eggs in many animals by a number of workers. Some of these degenerating oocytes are in mature follicles, filled with liquor folliculi, others are in follicles with a small antrum, or none at all, and others have only a single or double layer of granulosa cells around them. It is only in the larger follicles that the theca interna can be distinguished from the theca externa; in the smaller follicles the membrana granulosa is immediately surrounded by a thin fibrous sheath exactly similar to the theca externa of the mature follicle, in which no glandular elements of the theca interna can be distinguished. At one week after irradiation some of these follicles show signs of degeneration. At three weeks all the follicles are degenerate and some in advanced stages. Five weeks after irradiation and at all subsequent stages the degeneration of the follicles is complete and they are only represented by the remnants of the zonæ pellucidæ, each enclosed in a small cavity. Text-figs. 1 to 6 represent stages in this degeneration process. They were drawn with the aid of the camera lucida and are only slightly diagrammatic. Text-fig. 1 represents an approximately normal oocyte which has completed the growth phase, but the follicle is still small without an antrum. The oocyte degenerates first, the nucleus becoming crenated and losing its affinity for stain, the cytoplasm becoming shrunken and vacuolated and the zona pellucida puckered. At this stage a cavity forms between the oocyte and the membrana granulosa which, after chrome-osmic fixation, can be seen to contain droplets of fat, presumably arising from the breakdown of the oocyte. Text-fig. 2 represents this stage.



Text-Figs. 1–6.—× 560 approx.

The zona pellucida becomes more crenated as the cytoplasm of the oocyte breaks down and is absorbed. Finally the oocyte is only represented by the puckered and shrunken zona pellucida. By this time the cells of the membrana granulosa are degenerating also, with shrunken vacuolated cytoplasm and pycnotic nuclei, and individual cells have become detached and are free in the cavity of the follicle as in text-fig. 3. The break up of the membrana granulosa appears to be followed by that of the theca interna in those larger follicles in which it is differentiated as a definite layer. In these cases the degenerating theca interna cells become detached and merged with the mass of broken-down granulosa elements in the cavity. At a slightly later stage the follicle is represented by a few very pycnotic nuclei and cytoplasmic remnants, surrounding the old zona pellucida, in a cavity enclosed by the fibrous theca as in text-fig. 4. Gradually the contents of the cavity, with the exception of the old zona pellucida, are completely absorbed, as shown in text-fig. 5 and Plate 4, fig. 3. The zona pellucida appears extremely resistant and in many cases is still to be seen in the cavity a long time after irradiation. In some cases the zonæ appear to become broken down at last, and the old follicle is only represented by a more or less collapsed cavity containing a slight, weakly eosinophil coagulum, as shown in text-fig. 6. In other cases even these have vanished and no traces whatever of the old follicles remain. At all stages in this process the cavity is enclosed by the thin fibrous coat, which exactly resembles the theca externa of the mature follicle and the primitive theca of the smaller follicle before it has differentiated into externa and interna. In no case are any glandular elements, that might be considered as typical theca interna cells, found in this wall or outside it. This degeneration process is similar to that which can be observed in any adult mouse ovary. In M 39 and 1*a* the fibrous cells of some of these old follicles seem to have penetrated the membrana granulosa and to have formed the wall of the cavity within it. Outside the wall the membrana granulosa cells appear to persist, and to be changing and coming to resemble those that are being proliferated from the epithelium in the form of cords. They appear to be growing and forming a small zone around the old cavity, which stains slightly more deeply than the surrounding old stromal tissue. It is therefore probable that these cells, at least in some cases, contribute to a small extent to the tissues of the sterilised gonad, but it is also probable that in later stages they become atrophic, with other derivatives of the original stroma, and are pushed into the medullary zone by the growth of the epithelial cords. On this account, and because the follicle as such has disappeared, we can see no reason for considering them as follicle cells or for attributing to them the same physiological functions.

Sometimes mature, or nearly mature, follicles undergo a different process of atresia after irradiation. In these the oocyte breaks down as described already, but the cells of the membrana granulosa and theca interna, instead of degenerating, grow into the antrum, in the same manner as after ovulation, and form a luteal-like structure with a small central cavity, often containing the remains of the zona pellucida of the old oocyte. The central cavity, zona pellucida remains, and their somewhat smaller size serve to distinguish these corpora lutea atretica from the corpora lutea vera. A more detailed account of these luteal structures, and their origin will be reserved, for convenience, for a subsequent paper. M 39, 40 and 41 exhibit successive stages in their formation and they are also present in 42, 8, 12, 26, &c.

The inter-follicular tissue.—The inter-follicular tissue undergoes little change during the first fortnight after irradiation. The destruction of the follicles results in a sudden diminution in size of the whole ovary and a loosening of the cortical tissue owing to the removal of the pressure exerted by the follicles. At about fourteen days after irradiation changes become observable in the periphery of the gonad. The epithelium becomes cubical and begins to proliferate cells beneath its surface, between it and the tunica albuginea. These cells are at first typical epithelioid elements. At the same time the tunica albuginea becomes rather better developed and thickened. As the germinal epithelium continues to proliferate, the resulting cells press upon the tunica albuginea and begin to grow into the cortex in the form of thick finger-shaped cords carrying a layer of the cells of the tunica albuginea with them, which form a thin fibrous sheath to these cords (shown in plan in Plate 4, fig. 2). Three weeks after irradiation these cords are at this stage and the old inter-follicular tissue is showing signs of being in an unhealthy state and of atrophy. At four weeks the cords are well developed, the epithelium is rapidly proliferating and the tunica albuginea is much disturbed by the ingrowths. At all later stages the cords form the major portion of the ovary (text-fig. 7 and Plate 2, figs. 1, 3, and 4), and have pushed the old inter-follicular tissue and the cavities representing the old follicles before them towards the medullary region of the ovary (Plate 4, fig. 3). Some elements of the old inter-follicular tissue and a few of the cavities may persist, either pressed between the cords or in the medullary zone.

The growth of the cords is in part due to the continued proliferation of the germinal epithelium and in part to the mitoses of their cells. As the cords grow in, the cells composing them gradually lose their epithelial character, their nucleus enlarges, the cytoplasm increases, and fat globules become demonstrable in them. When they have reached the hilar region of the medulla, which is always composed of loose connective tissue and blood vessels, growth ceases. The germinal epithelium stops proliferating and becomes thin and inactive; the tunica albuginea rearranges itself in the form of a thin fibrous layer immediately beneath the epithelium; and the cells of the cords stop dividing. Some connective tissue cells, apparently from the sheaths of the cords which originated from the tunica albuginea, seem to grow in between the cells of epithelial origin. The latter cells enlarge considerably, the nuclei growing and the cytoplasm becoming even larger in proportion.

At this stage the cytoplasm stains poorly and the nuclei well. Fat fixatives reveal the presence of fat globules in increasing amounts in them. These changes result in the proliferated tissue after a time coming to resemble very closely luteal tissue (Plate 5, fig. 1). The fact that they are still in the form of finger-shaped cords, with only thin connective tissue sheaths, serves to distinguish them histologically from corpora lutea vera or corpora lutea atretica, which are much larger, spherical and with a more definite fibrous coat. Cytologically, the true luteal cells stain more deeply, and their cytoplasm never becomes so fat-laden as that of the cells of the cords in advanced stages. The resemblance to true luteal tissue is especially striking in 7*a* and *c* (Plate 5, fig. 2), the former of which did not exhibit a cycle for 57 days and the latter for 74 days. A fuller description and comparison of these cells will be given in a subsequent paper.

The ovaries of some animals appear to remain permanently at this stage and never to go beyond it, however long they are kept. M 7*a*, 7*c*, 10, 11, 21, &c., are examples of this.

The ovaries of other animals, having arrived at this state, undergo still further changes. The germinal epithelium becomes cubical, thickened and takes the stain more deeply, and the tunica albuginea also becomes thickened. The epithelium then begins rapidly proliferating cells beneath its surface. This second post-irradiation proliferation results in the production of small masses or cords of cells which become separated from the epithelium almost as soon as they are formed and are consequently almost spherical or slightly elongated. Their formation can be observed actually in the thickened epithelium, where a few epithelial cells group themselves together and become separated from the under surface of the epithelium. These, becoming pushed in by the proliferation of more, force themselves through the tunica albuginea, which contributes a connective tissue sheath of fibroblasts to each, into the cortex of the ovary (Plate 4, fig. 1). After separation from the germinal epithelium, these cords grow partly by the enlargement of the epithelial cells but chiefly by their multiplication. They may attain a diameter of about 0·1 mm., but never seem to grow much beyond this size. In the more advanced cases the nuclei of some of the epithelial cells in these cords become slightly enlarged and, exhibiting mitotic figures, are suggestive of the spermatogonia in embryonic testis tubules, while the other cells, with smaller nuclei, resemble Sertoli cells in a remarkable manner.

The second phase of proliferation appears to extend over a long period, as none of the material shows it to have stopped completely, although some ovaries, especially those of M 6*e* (Plate 2, fig. 2, and Plate 3, fig. 1), have the cortex almost exclusively composed of the resulting cords or tubules.

The cords formed at the beginning of the second proliferation are somewhat different from the later formed ones, and are more or less intermediate in character between them and the cords of the first proliferation (Plate 2, fig. 4). The connective tissue sheath of each is thinner than the later formed ones, the cells grow larger and mitoses are more frequent, and the tubules as a whole have not such a strong affinity for the hæmatoxylin. These are well shown in M 15. Each of the later formed tubules, which do not grow very large, has a very well-developed fibrous sheath. In the older ones a distinct tendency for the cells to arrange themselves as a lining epithelium, with a distinct lumen in the centre containing a few free cells, is observable (Plate 3, fig. 1), and is especially well shown in M 6*e*, in every way the most advanced stage so far produced. Such an arrangement in section affords a striking resemblance to embryonic testis tubules, but in no case have undoubted reduction figures been observed. Indeed, in M 6*e* the cells of these tubules, if structures that vary from being three or four times as long as broad to spherical may be designated as such, all resemble the Sertoli cells of undescended testes and no cells suggestive of germ-cells are present.

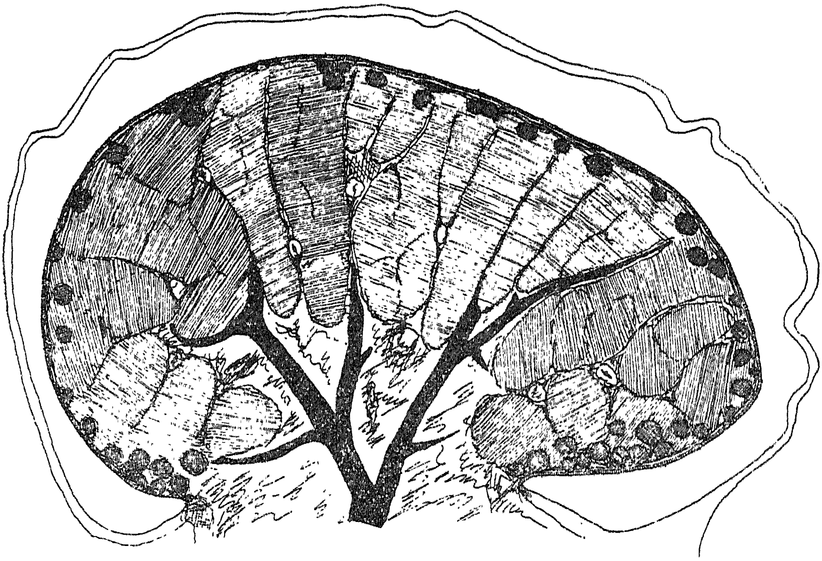
The production and growth of these second proliferations results in the pushing in towards the centre of the gonad of the tissue of the first proliferation. In most cases this only results in a peripheral zone of the former being formed around the latter, but in M 6*e* it has progressed so far that almost all the gonad is formed of the second proliferations, and only compressed remnants of the cords of the first proliferation can be discerned in the small central medullary region (Plate 2, fig. 2).

The chrome-osmic methods reveal the presence of small sub-equal globules of fat in clusters in the cytoplasm of the cells of the tubules of the second proliferation. This is quite unlike the irregular distribution of fat in large and small vacuoles in the cells of the first proliferation of cords. The germinal epithelium in the region of the hilus appears to proliferate more actively than that over the remainder of the surface of the gonad. In consequence, pockets of tubules of the second proliferation can often be seen in the hilar regions when they are seldom more than one deep around the rest of the periphery of the gonad.

(*b*) *Topographical Changes following Irradiation.*

The degeneration of the follicles following irradiation results in a marked diminution in volume of the ovaries, which never regain their normal size. The actual size of the gonads of the irradiated females described in this paper varies considerably, but the volume of one when adult rarely exceeds one-third that of the average ovary of a normal animal. In some cases it is very much smaller and may occasionally measure little over 1 mm. in greatest diameter.

The blood supply to many of these ovaries is markedly rich and it cannot, therefore, be urged that the decrease in volume, or other condition observed, is due to malnutrition. It also serves to account for the rapid absorption of the degenerating tissues.



TEXT-FIG. 7.

The topographical relations of a typical adult ovary irradiated at weaning time are shown to scale, somewhat diagrammatically, in text-fig. 7. It can be seen from this that the loose tissue of the hilar region is small in extent. The enlarged cords of the first proliferation comprise the greater part of the ovary. Between them, chiefly at their distal ends in the central region, are a few cavities in the scant connective tissue, representing the sites of old follicles and remnants, which, with the tissue of the hilar region, represent all that remains of the original stroma. The blood vessels, shown in black, are distended to the extent typical of the hyperæmic ovaries. The germinal epithelium is represented as beginning the second proliferation of cords, a small number of which are scattered round the periphery, in and beneath the tunica albuginea, and are more numerous in the region of the hilus. The intact capsule is represented in outline. In M 6*e* the cords of the second proliferation form the major portion of the gonad, and are arranged in a zone around the first cords, many times thicker in proportion than in text-fig. 7.

(*c*) *Anomalous Structures found in Irradiated Ovaries.*

In M 41, 3*a* and 51, one or two large follicles filled with extravasated blood are present. These are obviously produced by the rupture of the capillaries of the theca occurring when the follicle starts to break down. In addition to these, cysts are found in several ovaries, *i.e.*, M 2*d*, 6*b*, 21, 49 and 51, and it seems probable that they have originated from degenerating follicles and that those filled with blood are early stages in their formation. The cysts vary from half the size to rather larger than a mature follicle and have no opening. They are more or less spherical, and, with the blood-filled cavities, are chiefly situated in the hilar region. The cysts are lined by a thin, single-layered cubical epithelium, surrounded by a thin, fibrous, connective tissue coat. There is little or no coagulum in them.

Another kind of anomalous structure, of which we can suggest no explanation, is found in M 42, 15 and 16. We refer to the epithelial tubes, especially well developed in the last-mentioned case, which ramify deep into the gonad and open on its surface (Plate 2, fig. 4, and Plate 5, fig. 1). The lumen of these is clear and the epithelium lining is cubical and resembles the germinal epithelium with which it is continuous. Surrounding the epithelium is a thin layer of connective tissue resembling and continuous with the tunica albuginea. In M 42 and 15, the canals are similar to those in M 16 and open on to the surface, but they do not extend far into the ovary. It would appear that they are formed by invaginations of the germinal epithelium, carrying in with them a surrounding layer of tunica albuginea cells to form a sheath.

IV.—Discussion.

The regulation of the œstrous cycle.—The periodic occurrence of œstrus has usually been attributed to the cyclic appearance of mature follicles or to the subsequent appearance of corpora lutea. The arguments for and against both hypotheses have been discussed elsewhere (8) and need not be repeated here. It has long seemed difficult to attribute the initiation of the œstrous cycle to the corpus luteum, and recently it has generally been supposed that the Graafian follicle is the controlling factor. It has, however, been demonstrated in a previous paper (Parkes, 8), and in the present, that the œstrous cycle is maintained in the mouse by the irradiated ovaries, in which there is no rhythmical development of Graafian follicles or corpora lutea. Therefore, the arguments that one or both of these structures are essential for the cyclic production of œstrus are invalidated.

The vaginal contents of 29 of the 47 animals described in this paper were examined regularly for a longer or shorter period. Of these 29 animals 28 were observed to have from 1 to 14 œstrous periods. Double ovariotomy was performed on 9 of these, and in all cases was immediately followed by complete cessation of the œstrous cycle. We believe that this demonstrates that the irradiated ovary, though lacking follicles or rhythmically developing corpora lutea, is the organ producing the stimuli required for the occurrence of the œstrous cycle.

It only remains for us to consider in the present paper what constituents of the irradiated ovary are responsible for the elaboration of the œstrus-producing hormone, for which the name "œstrin" is elsewhere suggested (9). There are two possibilities, (*a*) that the persistent remains of the ovarian tissues present before irradiation are responsible, and (*b*) that new tissues proliferated after irradiation assume an œstrus-producing function.

In regard to the first possibility, it is difficult to attribute the entire production of œstrin to the old stromal tissue remnants which are represented in the adult by a few connective tissue elements in the region of the hilus and often appear to be entirely absent. No remains of true corpora lutea can be present, as all the animals described in the present paper were irradiated before puberty. The alternative is that the remnants of the follicles are responsible. All the oocytes degenerate so completely that they need not be further considered. Some of the larger follicles produce corpora lutea atretica, in the formation of which both theca interna and membrana granulosa elements participate, and which may persist for an indefinite period. If these structures are present at all, they are fully formed 6 weeks after irradiation. It will be shown elsewhere that true luteal tissue persisting in the mouse ovary for more than three weeks becomes functionless and inactive so far as the œstrous cycle is concerned. No difference can be detected between the œstrous cycles observed in those animals with corpora lutea atretica and those without. This suggests that they are not essential, and are probably functionless as regards the regulation of the œstrous cycle. The anomalous cystic follicles are present in so few of the animals that they are not worthy of consideration in this respect. With the exception of these structures, no liquor-filled cavities, like the antrum of a maturing follicle, were observable in any of the ovaries. In all the other follicles the membrana granulosa degenerates completely, as does the theca interna in the larger ones, where it is differentiated from the theca externa. There remain only the elements of the undifferentiated thecæ of the small follicles and the thecæ externæ of the large follicles, which are indistinguishable from each other. These remain unaltered as a few fibrous cells, surrounding the cavities which represent the locations of the old follicles, and which have no appearance of having a glandular function. In M 39 and 1*a*, however, cells possibly derived from the membrana granulosa appear to be growing and proliferating. Their derivatives resemble the epithelial elements proliferated from the germinal epithelium soon after irradiation. They must be considered in the second category, as constituting part of the new tissue proliferated after irradiation.

The alternative, that the new tissues proliferated after irradiation are responsible for the regulation of the œstrous cycle in the animals described, has more appearance of being the true explanation. As mentioned above, at least a small part of these tissues appears in some cases to be contributed by the proliferation of the membrana granulosa elements. This constituent, however, appears to be absent in many cases and is negligible in others. Although it may take part in the production of the œstrin when it is present, it cannot be regarded as an essential source. We therefore look to the cords proliferated from the germinal epithelium, together with their connective tissue sheaths, as probably the chief source of œstrin in the irradiated ovary. Further, it is very significant that the œstrous cycle charts reveal no distinctions between the animals whose ovaries showed only the first proliferation, and those in which the ovaries were found to contain both first and second. Again, the only animal that had little tissue of the first proliferation and much of the second, M 6*e*, was never observed to have an œstrous period, though examined for 134 days consecutively. These facts clearly suggest that the cords of the first proliferation of the germinal epithelium actively produce œstrin, and that those of the second probably do not. It is interesting that almost immediately after their separation from the germinal epithelium, the cells of the first proliferation become enlarged and glandular in character, and laden with fat globules. This may be connected with the fact that œstrin is a fat-soluble substance, and that it might be expected to be localised within the cells in fat globules. It is remarkable that careful study of the ovaries described, although taken from animals killed at all stages of the œstrous cycle and fixed in many cases for fat-content, revealed no cyclical changes in their cells or tissues, and no periodic increase or decrease in the fat-content. Either the oestrus-producing substance is elaborated rhythmically and we were unable to detect it by histological methods, probably owing to its small amount, or it is produced continuously and the rhythm is accounted for by the periodic attainment of a threshold value. The latter hypothesis is supported by the fact that œstrin can apparently be extracted from ovaries at any stage of the œstrous cycle, but it postulates its subsequent exhaustion or destruction, of which we have no knowledge at present.

The tissue of the first proliferation often becomes like luteal tissue, the epithelial cells becoming like luteal cells, with connective elements of the sheath growing in amongst them like the thecal cells of the corpus luteum. In advanced stages this tissue becomes fatty-degenerate, as does the corpus luteum. We reserve a fuller discussion of this similarity to a subsequent paper dealing with a series of experimental animals irradiated at or before birth. M 7*a* and *c* probably exhibit the most advanced stages of fatty degeneration of this tissue so far observed, and it is noteworthy that both exhibited an œstrous period at first, which stopped later and was not observed for several weeks before they were killed. This supports the view that the tissue of the first proliferation is responsible for the production of the œstrous condition, and that when it becomes fatty-degenerate, the cycle stops.

We therefore conclude that neither the Graafian follicle nor the corpus luteum is essential for the regulation of the œstrous cycle in the mouse, and that, whether or not any of the cells of the former produce œstrin in the normal animal, other cells do so in the irradiated mouse. It is worth noting, however, that the post-irradiation proliferations have the same origin as the follicle cells, in so far as they both come from the germinal epithelium. The tissues of the normal ovary must produce œstrin, and therefore any remnants which persist in the irradiated ovary may do so too, although in many cases they appear inadequate in amount to account for the whole production. It will be shown in a later paper, however, that irradiation of the adult female causes no hiatus in the cycle, and, since the production of the first proliferation must of necessity take some little time, it is necessary to suppose that in these circumstances the old tissue of the irradiated ovary can control the cycle.

The Morphological Significance of the Cords.—The epithelial cords of the post-irradiation proliferations tempt comparison with those which, during embryonic and pre-pubertal life, normally take part in the organogenesis of the mammalian gonads.

The cords of the first post-irradiation proliferation at first resemble the medullary cords of the indifferent gonad of the mouse embryo at 12 days *post coitum.* Like these, they are blunt, finger-shaped cords of epithelial cells, with connective coats derived from the albugineal layer. They are, of course, more numerous, and grow larger, as would be expected on account of the greater volume of the irradiated gonad. However, the medullary cords always contain germ-cells with larger nuclei, which are distinguishable from the first, whereas in the first post-irradiation cords these are absent. In a few cases, the latter in their later stages exhibit some cells, each with a larger nucleus and cytoplasm, which do suggest germ-cells, but are probably produced as a phase of fatty degeneration. Nevertheless, the similarity of these two sets of structures is significant, although it cannot be pressed far.

The cords of the second post-irradiation proliferation suggest even more interesting comparisons. Their origin, like that of the first proliferation, is similar to that of the medullary and cortical cords of the normal ovary and to the spermatic tubules of the testis. They resemble structures described and figured by various authors (Hartmann, 7) in the mammalian ovary as anovular follicles. Their origin from the epithelium, their nearly spherical shape, and their subsequent growth up to a certain limited size, are points which support such a view. On the other hand, they are not formed around a definite pre-existing germ-cell, as are all normal follicles; indeed, whenever a normal follicle loses its oocyte, by degeneration or expulsion, it degenerates completely or else forms a corpus luteum atreticum. Further, their central cells never closely resemble membrana granulosa cells, they never form a liquor-filled antrum, and no layer like the theca interna is ever differentiated. Not only do they probably take no part in producing the œstrous cycle, but their growth and development are entirely independent of it, whereas follicular growth is invariably synchronised with the cycle in normal animals. Such evidence as there is, therefore, seems to be against the view that they are anovular follicles.

The resemblance of these cords to a number of structures described and figured in the ovaries of various mammals by many authors as spermatic tubules is remarkable. A comparison of our figures (Plate 3, fig. 1, etc. with those of the gonads of free-martin cattle of several authors (Willier, 14; Chapin, 5, etc.) will show how closely similar the second post-irradiation cords are to the sex-cords of the free-martin. We believe that this similarity is of great importance. The fact that the free-martin has undoubted male characters, as well as sex-cords like embryonic seminiferous tubules, affords strong evidence that the structures in question are really spermatic in nature. Hammond has described and figured (6) similar structures in the ovary of an inbred rabbit. The striking similarity of the structures in the irradiated ovaries to these spermatic tubules, as he considered them to be, led us to believe that they were the same. We are indebted to Mr. Hammond for allowing us to examine and compare his material, and we are satisfied that the tubules he has described correspond exactly to our second post-irradiation cords. The latter also resemble, as shown in the description, the spermatic tubules of the 13-day mouse embryo, but lack the primordial germ-cells. They are also like the sterile spermatic tubules of undescended testes and of certain sex-intergrade gonads, both of which are often devoid of all traces of germ-cells and are composed of Sertoli-cells within the fibrous wall. These however, unlike the embryonic tubules, are usually more adult in structure and are long and coiled.

On the other hand, the second post-irradiation cords have not been observed by us so far to develop beyond the stage of M 6*e*, nor do they contain undeniable germ-cells, although in some cases cells suggestive of spermatogonia are present. Further, they are always short or spherical in form, and never long and coiled. This last point is not, however, decisive, as the same applies to the spermatic tubules of the embryo immediately after their separation from the germinal epithelium. Their subsequent tubular form is attained by growth within the gonad.

We feel confident that the cords of the second post-irradiation proliferation in the mouse are morphologically the same as those described as spermatic tubules by Hammond in the rabbit ovary, and are probably the same as those described by various authors in the free-martin. We do not feel, however, that the data are sufficient to prove that they are or are not spermatic in nature, but what evidence there is seems to favour this hypothesis. If so, the initial stages of sex-reversal can be produced experimentally in the mouse by the action of X-rays on the ovary.

Summary.

1. The ovaries of 47 female mice, exposed to a full sterility dose of X-rays at 3 weeks old, are described. The œstrous histories of most of these animals are recorded elsewhere (8).

2. Irradiation is followed by the degeneration of all the oocytes.

3. The membrana granulosa and the theca interna, in those cases where it is differentiated, degenerate also. Finally, the old follicles are only represented by small cavities containing zona pellucida remnants.

4. In a few cases the larger follicles (*a*) become filled with blood and form cysts, or (*b*) the cells of the theca interna and membrana granulosa grow and, invading the antrum, form a corpus luteum atreticum.

5. These corpora lutea atretica persist indefinitely, but are shown to have no influence on the œstrous cycle.

6. Simultaneously with these changes the old inter-follicular tissue atrophies, and the germinal epithelium proliferates epithelial cords.

7. In the adult animals the ovaries are composed almost entirely of this first proliferation. It tends to become like luteal tissue in some cases.

8. In many cases a second proliferation from the germinal epithelium follows. This consists of small, spherical or slightly elongated cords. These cords resemble the so-called spermatic cords described in the ovaries of inbred rabbits by Hammond (6) and of free-martin cattle by a number of authors. They also resemble structures described by many authors as anovular follicles.

9. The cords of the second proliferation appear to have no effect on the œstrous cycle.

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DESCRIPTION OF PLATES 2–4.

Guide Letters.—*T.A.*, Tunica albuginea. *G.E.*, Germinal epithelium. *B.V.*, Blood vessel. *F.C.*, Cavity representing old follicle. *M.R.*, Loose medullary region. *E.C.*, Epithelial canal. *C1*, Cord of first proliferation. *C2a*, Intermediate type cord of second proliferation. *C2b*, Cord of second proliferation.

Plate 2.

Fig. 1.—M 20 showing maximum development of the first proliferation arranged in thick finger-shaped cords. × 50.

Fig. 2.—M 6e showing extreme case of the development of the cords of the second proliferation and almost total absence of the first proliferation. × 70.

Fig. 3.—M 50 showing peripheral zone of cords of second proliferation and cavities representing old follicles in the centre of the tissue of the first proliferation. × 40.

Fig. 4.—M 16 showing intermediate type cords of second proliferation in the periphery, somewhat luteal-like tissue of the first proliferation composing the major part of the ovary and an epithelial canal opening on the surface and penetrating to the centre. × 70.

Plate 3.

Fig. 1.—M 6*e* showing portion of Plate 2, fig. 2, at higher magnification. × 350.

Fig. 2.—M 1*d*, extreme tangential section showing in plan cords of second proliferation pushing through the tunica albuginea. × 250.

Fig. 3.—M 36, 7 days after irradiation, showing absence of primordial follicles. The first proliferation has not started. × 150.

PLATE 4.

FIG. 1.—M 6*c* showing portions of the periphery of both ovaries; cords of the second proliferation are being formed and are pushing in the cords of the first proliferation. × 250.

FIG. 2.—M 7*a*, extreme tangential section showing in plan cords of first proliferation penetrating the tunica albuginea. × 280.

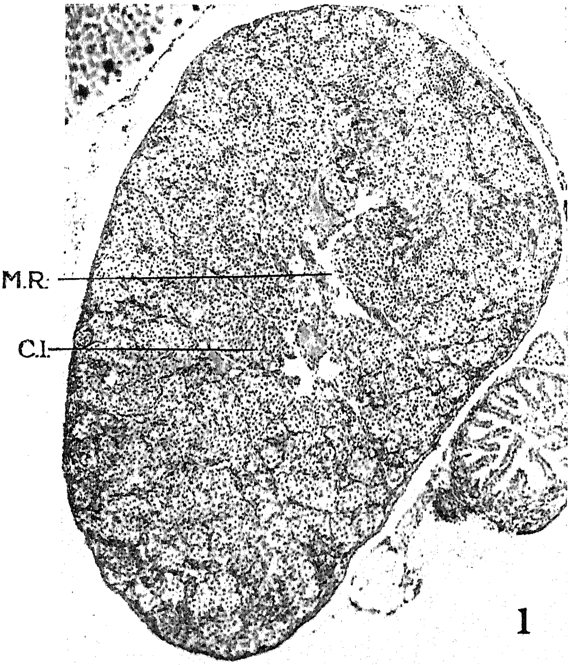
FIG. 3.—M 2*c* showing cavities containing zona pellucida remnants and necrotic follicle cells being pushed away from periphery by growth of the first proliferation. 200.

PLATE 5.

FIG. 1.—M 16 showing portion of Plate I, fig. 4, at higher magnification. The epithelial canal and somewhat luteal-like nature of the tissue of the first proliferation are apparent. × 310.

FIG. 2.—M 7*a* showing enlarged vacuolated tissue of the first proliferation, thin germinal epithelium and well-developed tunica albuginea. × 290.

We are indebted to Mr. F. J. Pittock for the photomicrographs reproduced in these plates.



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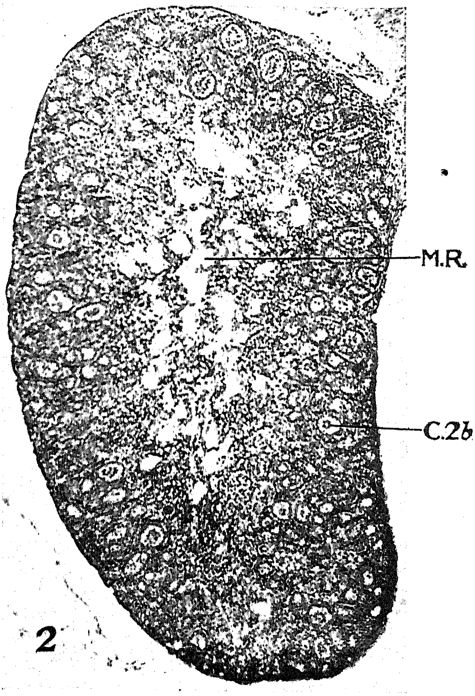

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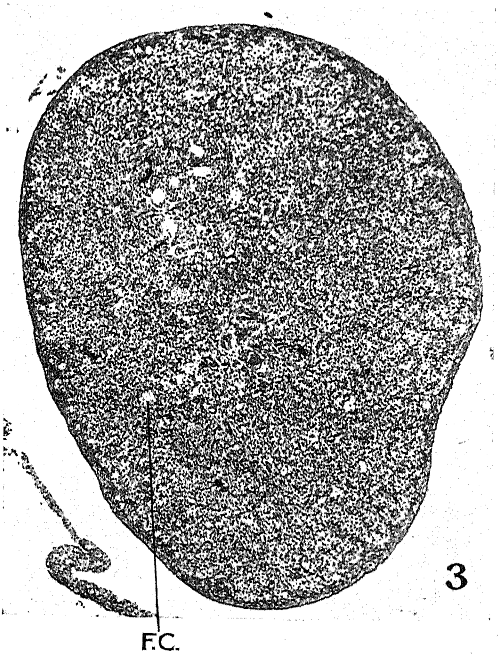

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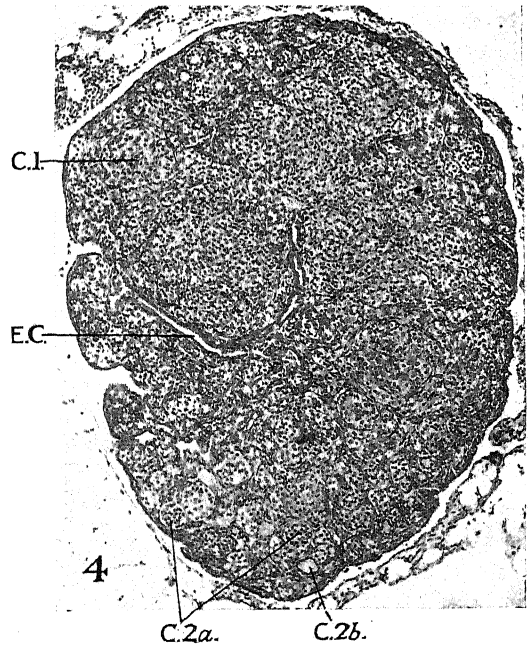

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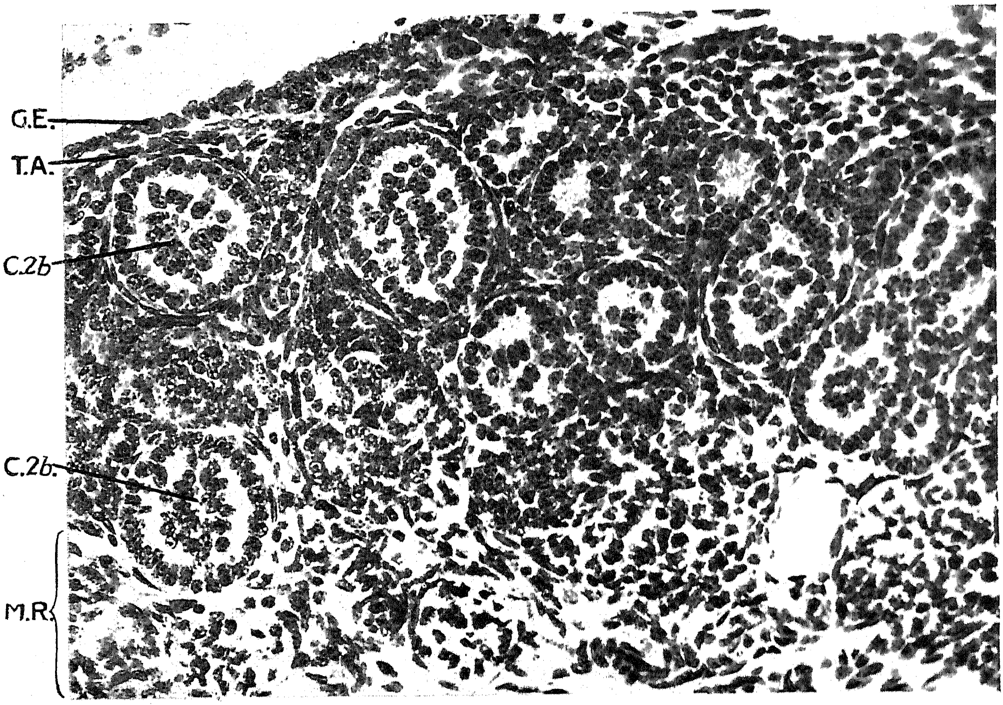

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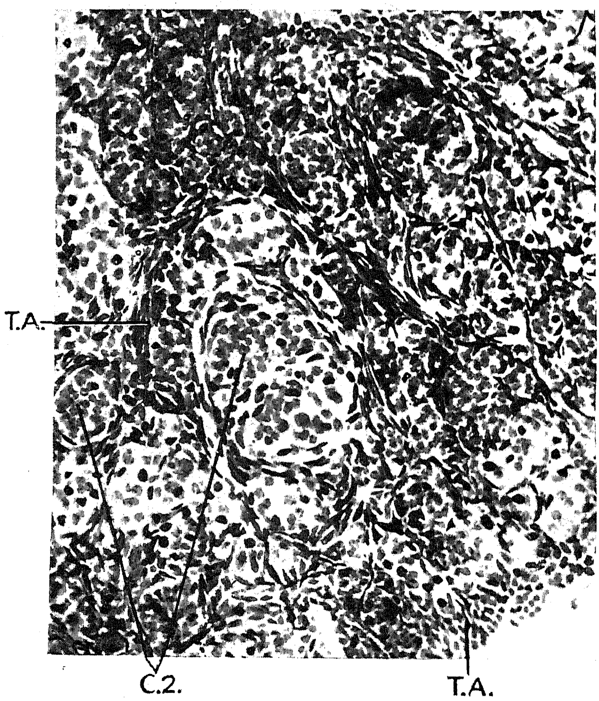

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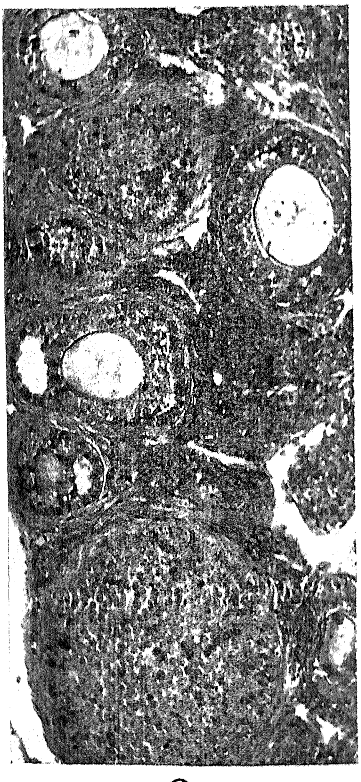

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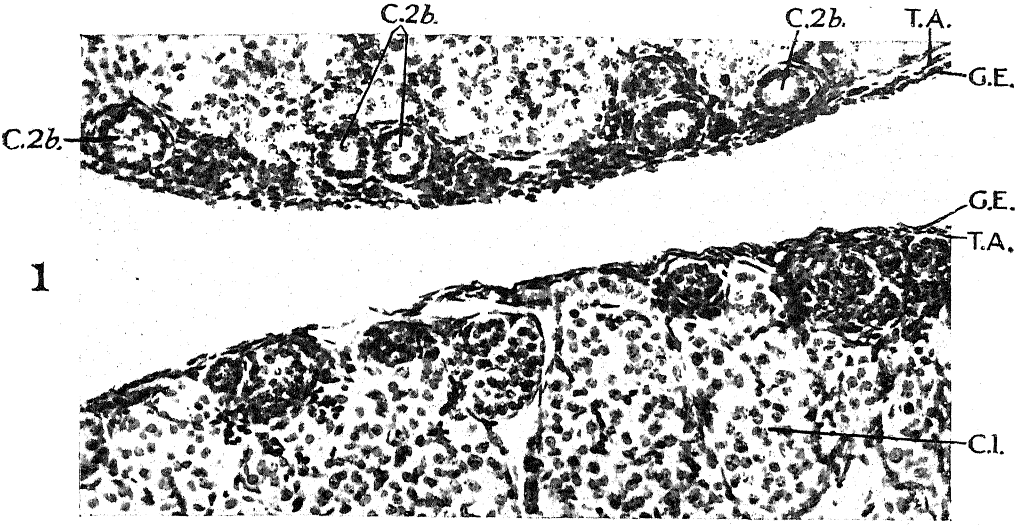

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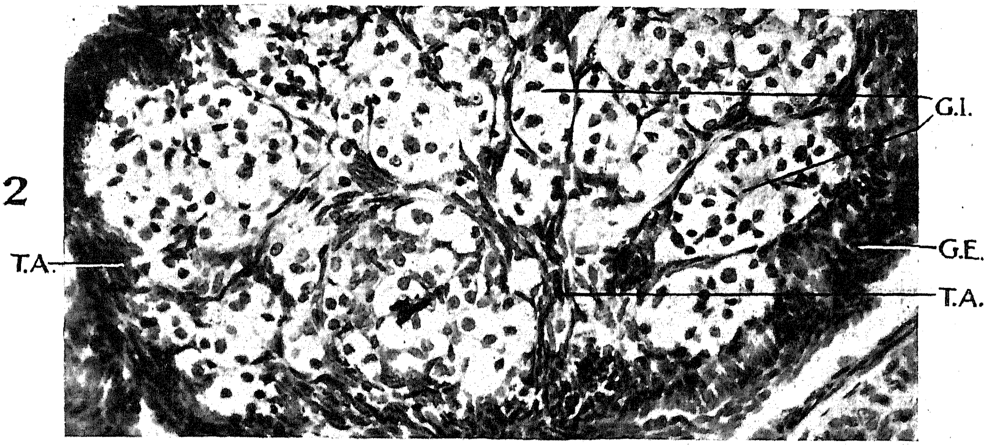

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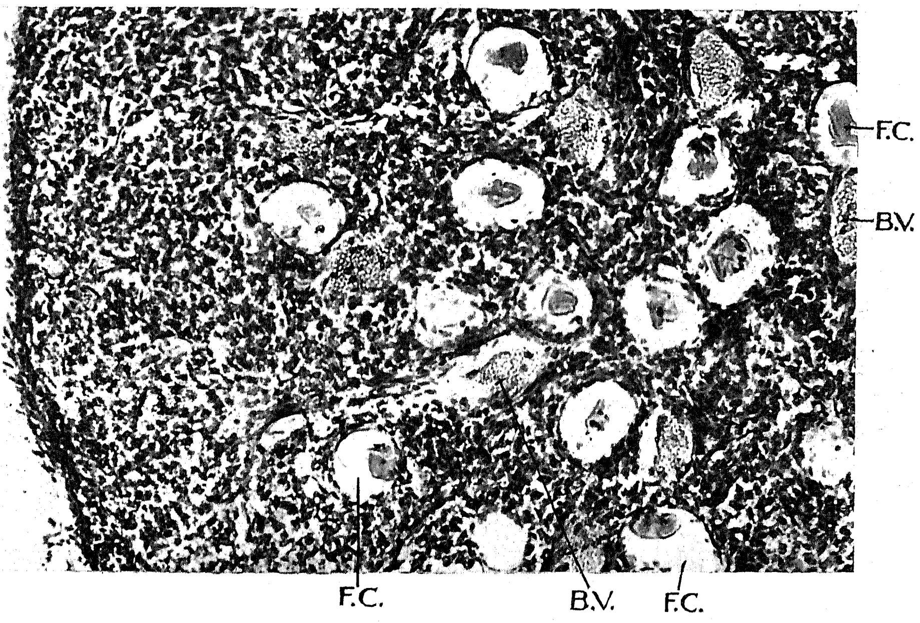

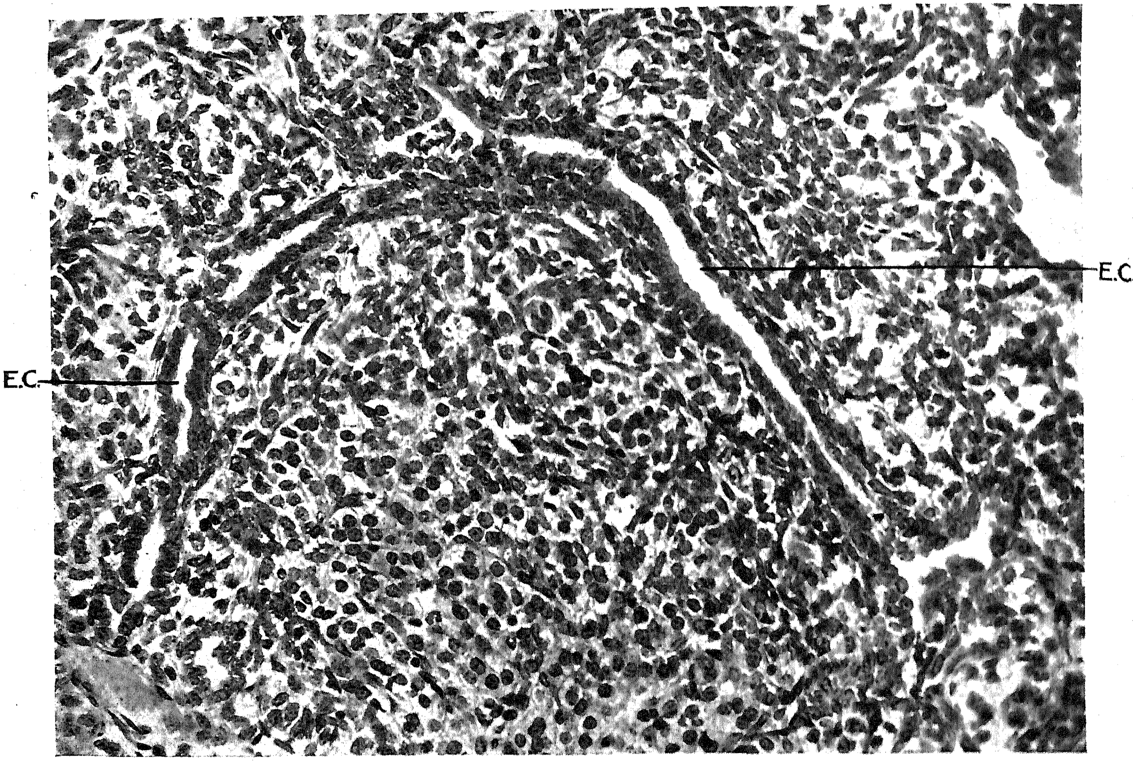

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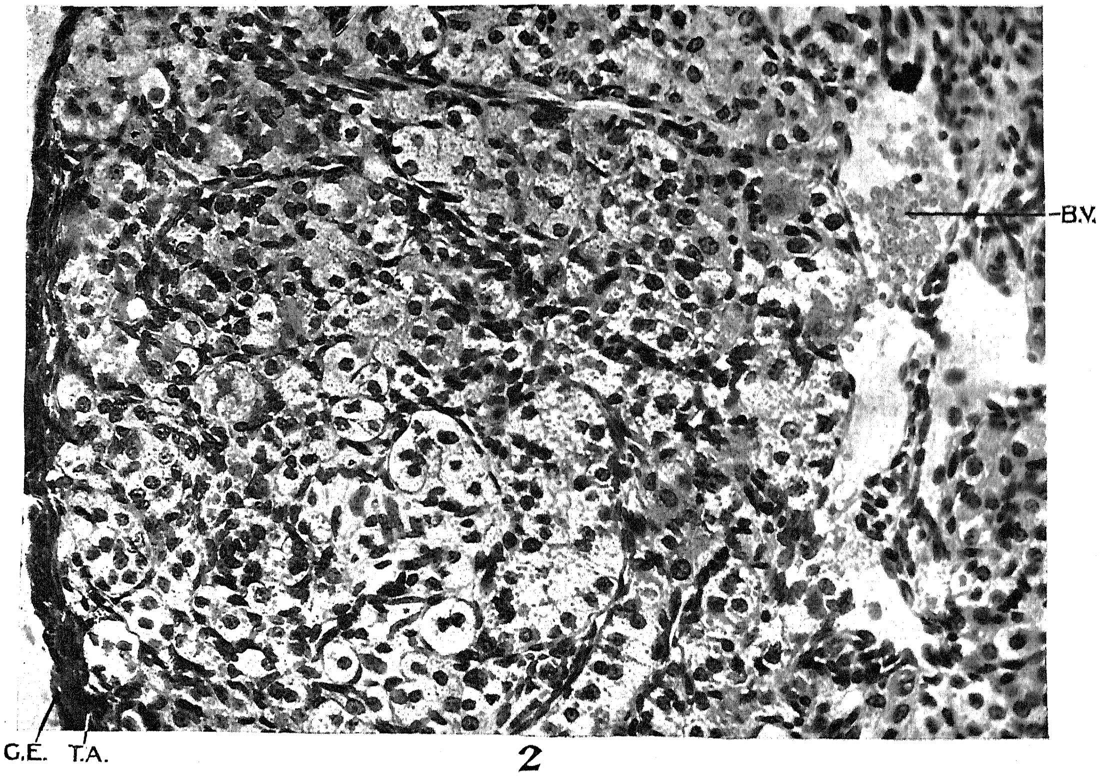


2

On the Mechanism of Oxidation-Reduction Potential.

By Malcolm Dixon, Ph.D. (1851 Exhibition Senior Student).

(Communicated by Sir Frederick Hopkins, F.R.S.—Received July 6, 1926.)

(From the Biochemical Laboratory, Cambridge.)

The phenomena of oxidation-reduction potential are becoming increasingly important in the study of biological oxidation processes. Unfortunately, however, the application of the results obtained from studies on potential to such processes has been somewhat hindered by the fact that the study of potential phenomena has up to the present been approached from a point of view which differs considerably from that of the more important theories of the mechanism of biological oxidation processes. The conceptions used in the development of the two subjects have been different, and the close connection between the two sets of phenomena has thereby been obscured. For instance, Wieland's theory, which has played such an important part in the development of the subject of biological oxidation processes, is founded on the conception of activation and transfer of hydrogen atoms, while Mansfield Clark, in his recent important and valuable studies on reduction-potential, makes use of conceptions such as electron activity, transfer of " electron pairs," and so on. This absence of " linking up " between the two sides of the subject has, in fact, been brought up as an objection to the Wieland view. For instance, Clark (3, 4) claims that the reduction-potential phenomena prove that the oxidation-reduction process consists essentially in the transfer of an electron pair from reductant to oxidant, and not in the transfer of hydrogen as assumed by Wieland. He regards this as a very serious objection to the Wieland theory, although possibly not sufficient entirely to disprove it. It must be pointed out, however, that the fact that the electron transfer hypothesis leads to correct results is no proof of its truth until the alternative hypotheses have been shown to yield incorrect results.

In the present communication it is shown that the Wieland view also leads to correct results ; in fact, it is possible to predict the reduction-potential phenomena from the work of Wieland. This at once disposes of one of the main objections to Wieland's theory and renders it possible to relate the mechanism of oxidation-reduction potential with that of biological oxidation-reduction processes.

The Hydrogen Electrode.

It will be necessary before considering the work of Wieland to make a few preliminary remarks about the hydrogen electrode, for reasons which will appear later. If a metal such as platinum is brought into contact with hydrogen gas the hydrogen is absorbed by the metal. The state of the absorbed hydrogen has been described in various ways—for instance, as a platinum-hydrogen alloy or a hydride, or a solid solution of atomic hydrogen, and in many other ways. These all seem to mean very much the same thing, and it is not necessary here to discuss which is the correct description. At any rate, we can picture the metal surface (with which we are alone concerned) as being more or less completely covered by hydrogen atoms loosely attached to the platinum surface.

The hydrogen in this state, which corresponds to the "active hydrogen" of Wieland, must not be regarded as atomic hydrogen, since it is in combination with the platinum. The attachment is, however, only loose, so that it does actually approximate to atomic hydrogen in behaviour. These hydrogen atoms are in equilibrium ((*a*) in the diagram, p. 60) with molecular hydrogen thus: $2H \rightleftarrows H_2$, and to any surface concentration of hydrogen atoms there corresponds a definite pressure of hydrogen gas. Thus the hydrogen-gas pressure can be used as a measure of the concentration of the hydrogen atoms. Applying the law of mass action to the equilibrium we get

$$[H]^2 = k_1[H_2]$$

where [H] is the surface concentration of hydrogen atoms and $[H_2]$ the concentration of molecular hydrogen; or since $[H_2]$ is proportional to P, the hydrogen-gas pressure, we may write

$$[H] = k_2\sqrt{P}.$$

Now, if the platinum surface is immersed in an aqueous solution the surface hydrogen atoms can also go into solution as hydrogen ions, and are in equilibrium with the hydrogen ions in the solution (equilibrium (*b*) in the diagram). They are thus electromotively active, and their electrolytic solution pressure will, of course, be proportional to their surface concentration; *i.e.*,

$$p = k_3\,[H] \quad \text{or} \quad p = k\sqrt{P}.$$

Substituting in the general electrode equation of Nernst, namely,

$$E = \frac{RT}{nF} \log \frac{c}{p}$$

we get

$$E = \frac{RT}{F} \log \frac{[H^{\cdot}]}{k\sqrt{P}},$$

where E is the absolute potential of the platinum, R is the gas-constant, T is the absolute temperature, n is the valency of the ion, and F is the Faraday constant; or, expressing the potential, as is usual, with reference to the normal hydrogen electrode, we may write

$$E_h = \frac{RT}{F} \log \frac{[H^{\cdot}]}{\sqrt{P}}.$$

This is, of course, the fundamental equation of the hydrogen electrode.

After these introductory remarks we now turn to the consideration of a certain aspect of the work of Wieland.

Wieland's Work on Platinum.

Wieland (6, 7) found that when platinum or palladium black, *i.e.*, a preparation of the metal in a state of fine division and presenting a large surface, was brought into contact with solutions of a number of oxidisable substances, such as hydroquinone, indigo white and other leuko-dyes, hydrazobenzene, sugars, etc., the platinum became laden with hydrogen and the hydroquinone, etc., was simultaneously oxidised. The hydroquinone, for instance, tends to pass over into its oxidised form, quinone, with loss of hydrogen atoms, which are taken up by the platinum. At the end of the experiment, the solution contains quinone, and on separating and heating the platinum hydrogen gas is given off.

On the other hand, Wieland showed that the hydrogen could again be taken up from the platinum by a number of reducible substances ("hydrogen acceptors"), such as quinone, methylene blue and other reducible dyes, etc., which thereby became reduced. The process in these cases is, in fact, reversible. Wieland used these facts in developing his theory of oxidation-reduction by hydrogen transfer.

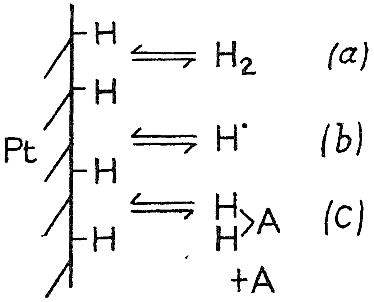
Now, on reading Wieland's work, one is struck by the fact that there would appear to be a general correspondence between the substances which give up hydrogen to platinum and those which give reduction potentials, and similarly those which take up hydrogen from hydrogenised platinum give oxidation potentials. For instance, hydroquinone, leuko-dyes, hydrazobenzene, sugars, etc., all give reduction potentials, while quinone, methylene blue, etc., give oxidation potentials. Aldehydes, on the other hand, were found by Wieland not to give up hydrogen to the metal, and they do not give any potential. This correspondence, although at present based upon somewhat limited data, suggested the view developed in this paper.

It appears then that the hydrogen atoms on the platinum surface are in equilibrium in yet a third way, which can be represented as

$$AH_2 \rightleftarrows A + 2\,H,$$

where H represents a hydrogen atom on the platinum surface (equilibrium (*c*) in the diagram).

We may represent the whole system diagrammatically as follows :—



where AH_2 represents some molecule analogous to hydroquinone.

Derivation of the General Reduction-Potential Equation.

We may now pass on to consider how these facts are capable of explaining the phenomena of reduction-potential. Of the three equilibria represented in the diagram, it is clear that (*b*) is the only one which can directly affect the potential of the platinum, since the others are non-ionic. We may regard the platinum as a hydrogen electrode in which the hydrogen pressure is controlled by equilibrium (*c*), or, in other words, by the amounts of oxidant and reductant in solution.

Now, if we increase the amount of reductant (AH_2) in solution, more hydrogen atoms will be transferred from this to the platinum, so that the surface concentration [H] will rise. In consequence, more hydrogen ions will pass from the electrode into the solution, and the potential of the electrode will become more negative. Conversely, an increase in the concentration of oxidant (A) will have the opposite effect, and the electrode will become more positive. An increase in the acidity of the solution will, of course, drive back hydrogen ions on to the electrode surface, and make it more positive, and *vice versa.* Qualitatively then at any rate the effects expected are in agreement with actual measurements of reduction-potential.

It may be mentioned here in parenthesis that, as there is a definite pressure of hydrogen gas in the platinum, there will always be a slight diffusion of this from the electrode into the solution. The hydrogen pressures are, however, in general very small, so that the amount diffusing will also be very small; and, moreover, there is good reason for believing that reaction (*c*), in the systems we are considering here, is rapid in comparison with the diffusion process, so that any effect of diffusion is negligible. We shall see later, however, that this is probably not always the case, but here we shall assume that the effect of such diffusion upon the hydrogen atom concentration is negligible.

We must now see how far the quantitative deductions on this view harmonise with the known facts of reduction-potential. Equilibrium (*c*) can be written :—

$$\mathrm{AH_2} \rightleftarrows \mathrm{A} + 2\mathrm{H},$$

where H denotes a hydrogen atom on the platinum surface. Applying the mass-action law to this we get :—

$$[\mathrm{AH_2}] = \mathrm{K}'[\mathrm{A}][\mathrm{H}]^2.$$

We saw earlier that

$$[\mathrm{H}] = k_2\sqrt{\mathrm{P}},$$

so that we obtain

$$\mathrm{P} = \mathrm{K}\frac{[\mathrm{AH_2}]}{[\mathrm{A}]}.$$

Now the hydrogen-electrode equation given above can be re-written

$$\mathrm{E}_h = \frac{\mathrm{RT}}{2\mathrm{F}}\log\frac{1}{\mathrm{P}} - \frac{\mathrm{RT}}{\mathrm{F}}\cdot p\mathrm{H},$$

where *p*H has its usual significance; and, substituting, we get

$$\mathrm{E}_h = \frac{\mathrm{RT}}{2\mathrm{F}}\log\frac{[\mathrm{A}]}{\mathrm{K}[\mathrm{AH_2}]} - \frac{\mathrm{RT}}{\mathrm{F}}\cdot p\mathrm{H}$$

$$= \frac{\mathrm{RT}}{2\mathrm{F}}\log\frac{1}{\mathrm{K}} - \frac{\mathrm{RT}}{2\mathrm{F}}\log\frac{[\mathrm{AH_2}]}{[\mathrm{A}]} - \frac{\mathrm{RT}}{\mathrm{F}}\cdot p\mathrm{H},$$

or, writing E_0 for the first (constant) term,

$$\mathrm{E}_h = \mathrm{E}_0 - \frac{\mathrm{RT}}{2\mathrm{F}}\log\frac{[\mathrm{AH_2}]}{[\mathrm{A}]} - \frac{\mathrm{RT}}{\mathrm{F}}\cdot p\mathrm{H}.$$

But this is the ordinary accepted reduction-potential equation for systems of the general type $\mathrm{AH_2} + \mathrm{A}$, for example, the hydroquinone-quinone system, etc.

Thus, starting from Wieland's observations, and making only the simplest assumptions, we arrive at the general reduction-potential equation, which is identical with that obtained on the electron-transfer theories. Had we known nothing of the reduction-potential phenomena we might, in fact, have predicted them from the experiments of Wieland.

The Effect of Ionisation.

In cases where one or both of the constituents of the system ionise, the equation must be modified to take into account the effect of the ionisation on the potential. For example, if the molecule AH_2 dissociates into A'' ions and hydrogen ions, the concentration of undissociated AH_2 molecules ($[AH_2]$ in the equation) will no longer be equal to the total substance AH_2 added.

Clark assumes that in all cases two hydrogen ions must be dissociated from the molecule, and that only the double anion A'' is active, giving up two electrons to the electrode. The opposite assumption will be made here, namely, that only the unionised molecules are active, and that they act by transferring two hydrogen atoms to the electrode in accordance with Wieland's theory.

The hydroquinone system may be taken as a typical example. Hydroquinone ionises as follows :—

$$H_2Q \rightleftarrows HQ' + H^{\cdot}$$

and

$$HQ' \rightleftarrows Q'' + H^{\cdot}.$$

By mass action

$$K_1 = \frac{[HQ'][H^{\cdot}]}{[H_2Q]} \qquad K_2 = \frac{[Q''][H^{\cdot}]}{[HQ']}$$

where K_1, K_2 are the respective ionisation constants. Writing S_0 and S_r for the concentration of total oxidant (quinone) and reductant (hydroquinone), respectively, using Clark's symbols, we have :—

$$S_0 = [Q] \qquad S_r = [H_2Q] + [HQ'] + [Q''].$$

Solving the three equations for $[H_2Q]$ we get :—

$$[H_2Q] = \frac{S_r}{\frac{K_1K_2}{[H^{\cdot}]^2} + \frac{K_1}{[H^{\cdot}]} + 1},$$

and substituting in the general equation given in the last section and re-arranging we obtain :—

$$
\begin{aligned}
E_h &= E_0 - \frac{RT}{2F}\log\frac{S_r}{S_0} + \frac{RT}{2F}\log\left(\frac{K_1K_2}{[H^{\cdot}]^2} + \frac{K_1}{[H^{\cdot}]} + 1\right) - \frac{RT}{F}\cdot pH \\
&= E_0 - \frac{RT}{2F}\log\frac{S_r}{S_0} + \frac{RT}{2F}\log\left(\frac{K_1K_2}{[H^{\cdot}]^2} + \frac{K_1}{[H^{\cdot}]} + 1\right) + \frac{RT}{2F}\log\,[H^{\cdot}]^2 \\
&= E_0 - \frac{RT}{2F}\log\frac{S_r}{S_0} + \frac{RT}{2F}\log\left[\left(\frac{K_1K_2}{[H^{\cdot}]^2} + \frac{K_1}{[H^{\cdot}]} + 1\right)[H^{\cdot}]^2\right] \\
&= E_0 - \frac{RT}{2F}\log\frac{S_r}{S_0} + \frac{RT}{2F}\log\,(K_1K_2 + K_1[H^{\cdot}] + [H^{\cdot}]^2).
\end{aligned}
$$

Now this last equation is the identical equation obtained by Clark (1) for this system, and verified experimentally. It will be seen, therefore, that the theory has again led to quantitatively correct results. Other and more complicated cases have also been worked out, but it is unnecessary to give details here, as it will appear later that the two theories are bound to give the same results. The above example will suffice to illustrate the method of procedure.

The Relation between the Two Theories.

A fuller comparison of the mechanisms by which a substance such as hydroquinone produces a reduction potential on the two theories will assist in making clear their relation to one another. On Clark's electron transfer theory the hydroquinone must first lose two hydrogen ions by dissociation as a dibasic acid. The doubly-charged anion then reacts with the electrode by transferring to it two electrons, thus becoming converted into quinone and making the potential of the electrode more negative. The balance of the equilibrium between the electrode and the hydrogen ions is thereby upset, so that in accordance with the hydrogen-electrode equation there is bound to be a passage of hydrogen ions from the solution to the electrode. The net result is therefore a passage of hydrogen ions and of electrons from the hydroquinone to the platinum.

On the present theory, on the other hand, there is a direct transfer of hydrogen atoms from the hydroquinone to the platinum, followed of course by a re-adjustment of the equilibrium with hydrogen ions.

Now a hydrogen atom consists of a hydrogen ion *plus* an electron, so that on thermodynamical grounds it is clear that the two theories must give the same results. The essential difference between the two mechanisms is that on Clark's view the hydrogen ions and the electrons are transferred separately, whereas here it is assumed that they are transferred together as hydrogen atoms.

It may therefore be concluded that the reduction-potential phenomena do not furnish a proof that the reaction takes place by the transfer of "electron pairs," since they follow equally well on the assumption of hydrogen-atom transfer. Clark's objections to the Wieland theory are therefore shown to be groundless.

It should be pointed out here in passing that this linking up not only enables us to explain the reduction-potential phenomena on the Wieland view, but it also enables us to explain the observations of Wieland on the electron-transfer view. For instance, on this view the addition of hydroquinone to a suspension of platinum black would produce a negative charge on the metal, by electron-transfer from the hydroquinone. This charge would cause the passage of hydrogen ions from the solution to the metal, and the disappearance of these ions would cause a further ionisation of the hydroquinine, and so on. The platinum would therefore become laden with hydrogen, which would have been derived from the hydrogen ions.

As the evidence at present does not appear to permit of a definite decision as to which mechanism is the true one, it would be dangerous to adopt one view to the exclusion of the other. The writer's personal view is that both actions probably take place. For instance in the case of hydroquinone, under conditions where ionisation is slight (*e.g.* in acid solution) the main action may be supposed to be a transfer of hydrogen atoms from the non-ionised molecules H_2Q; in alkaline solution, where it is mostly present as Q'' we may suppose a loss of two electrons, while at reactions where the singly charged ion HQ' is the main constituent the process may be assumed to consist in a loss of one hydrogen atom and one electron.

In certain cases the electronic theory appears to become somewhat far-fetched and artificial, particularly in those cases where no ionisation has been detected. For instance hydrazobenzene gives a high reduction-potential, but it has no acid properties whatever. Yet we have to assume, on the electronic view, that the only active form of the molecule is that in which, not one, but both of the hydrogen atoms attached to the nitrogen atoms have been dissociated as hydrogen ions. The amount of this dibasic acid ion must surely be infinitesimal under ordinary conditions. Clark himself has drawn attention to other similar cases. On the other hand, the Wieland theory would become equally artificial in any case where the ionisation of the hydrogen atoms involved was so strong as to be practically complete.

The rH Scale.

Clark (2) has recently introduced a very convenient scale of "reducing power," analogous to the *p*H scale of acidity. The physical basis and justification of this scale is brought out very clearly by the view put forward in the previous sections. According to Clark's notation the *r*H value of a solution is defined as log 1/P, where P as before is the pressure of hydrogen gas in a platinum electrode in equilibrium with the solution, which is, of course, calculated from the potential by means of the hydrogen electrode equation. The complete analogy between the *p*H scale and the *r*H scale will be clearly seen if the two sets of equations are worked out side by side.

By definition

$$r\mathrm{H} = -\log \mathrm{P} \qquad p\mathrm{H} = -\log [\mathrm{H}^{\bullet}].$$

The two equilibria are

$$\mathrm{AH_2} \rightleftarrows \mathrm{A} + \mathrm{H_2} \qquad \mathrm{AH} \rightleftarrows \mathrm{A}' + \mathrm{H}^{\bullet}.$$

Applying the mass law to these we get

$$\mathrm{P} = \mathrm{K}\,\frac{[\mathrm{AH_2}]}{[\mathrm{A}]} \qquad [\mathrm{H}^{\bullet}] = \mathrm{K}'\,\frac{[\mathrm{AH}]}{[\mathrm{A}']},$$

and taking logarithms we get

$$r\mathrm{H} = p\mathrm{K} + \log\frac{[\mathrm{A}]}{[\mathrm{AH_2}]} \qquad p\mathrm{H} = p\mathrm{K}' + \log\frac{[\mathrm{A}']}{[\mathrm{AH}]},$$

where A denotes either oxidant or acid molecule, *p*K means log 1/K, K is the oxidation-reduction equilibrium constant (expressing the affinity of oxidant for hydrogen), and K′ is the dissociation constant of the acid.

The last two equations represent the familiar dissociation or titration curve, and it can easily be shown that the laws which apply to *p*H in connection with acid-base equilibrium apply equally to *r*H in connection with oxidation-reduction equilibrium. The behaviour of strong and weak acids and bases, buffers, indicators, etc., all find an exact counterpart in oxidation-reduction equilibria.

Of course, a small *r*H value corresponds to a high reducing power, and in general it may be said that a system giving a small *r*H is bound to reduce one which has a larger *r*H, and *vice versa.* At any rate, this is true at the electrode surface, but we must not lose sight of the fact that potential measurements can only give information about what is happening at the electrode surface, and it is possible that in special cases the platinum might activate one of the constituents, or in other words act as a catalyst. In such cases the reaction might not proceed in the solution in the absence of a catalyst. Actually, however, it appears that in general reactions take place in solution in accordance with the *r*H data, and the practical value of, for instance, the " *r*H indicators " introduced by Clark depends upon this fact.

When the *r*H equals 0 the hydrogen pressure becomes atmospheric, and the gas begins to come off the electrode in bubbles. In the hydrogen electrode as ordinarily used of course the *r*H = 0. On the other hand, when the *r*H = about 41 the electrode evolves oxygen gas. There is always a balance between hydrogen atoms and hydroxyl groups at the electrode surface, and there is, in consequence, always an oxygen pressure in addition to the hydrogen pressure. Below *r*H 20 the hydrogen pressure will predominate, and above this value the oxygen pressure. We can, however, deal throughout with the hydrogen and neglect the oxygen, just as we speak of the *p*H of a strong alkali, although the hydrogen ion concentration is negligible in comparison with the hydroxyl ions.

Electrodes of different Metals.

Electrodes of different metals are, in general, found to give the same potential when immersed in solutions of reversible oxidation-reduction systems such as those we have been considering. Clark, for instance, has found that a quinhydrone solution gives identical potentials with electrodes of platinum, gold, palladium and mercury. This might perhaps be expected, for, although the affinities of the various metals for hydrogen differ very greatly, this may be supposed to affect all the equilibria in the same way, a metal with a high affinity for hydrogen being less ready to part with hydrogen atoms, either to the oxidant, or as hydrogen gas, or as hydrogen ions, than one with a low affinity. Thus, a given concentration of oxidant and reductant in the solution will be in equilibrium with a given hydrogen pressure and will correspond to a given electrolytic solution pressure whatever the metal; although, of course, the surface concentration of hydrogen atoms in equilibrium with these will naturally be greater in the case of a metal with a high affinity for hydrogen. Of course, it is assumed that the metal does not react chemically with the solution.

The hydrogen pressure therefore, and consequently the *r*H, is a true measure of the reducing power of a solution, being independent of the metal used.

In passing, it may be pointed out that it seems difficult to explain on the electronic theory why different metals should give the same potentials in such systems, as although the "electron activity" of the solution will remain constant that of the electrode will vary with the metal used.

The Sulphydryl System.

There is one reduction-potential system which differs fundamentally from the reversible oxidation-reduction systems we have considered hitherto, namely that formed by the sulphydryl compounds, cysteine and reduced glutathione, which was studied by Dixon and Quastel (5). These sulphydryl compounds are easily oxidised to the corresponding disulphide compounds, with loss of hydrogen atoms:

$$2\,\mathrm{R.SH} \rightarrow \mathrm{R.S.S.R.} + 2\mathrm{H}.$$

This system is anomalous in that (*a*) the potential percentage reduction curve is not S-shaped, but logarithmic, and (*b*) the oxidised form has no effect on the potential. The equation which Dixon and Quastel found to express the behaviour of the system was

$$\mathrm{E}_h = \mathrm{E}_0 - \frac{\mathrm{RT}}{\mathrm{F}} \log\,[\mathrm{R.SH}] - \frac{\mathrm{RT}}{\mathrm{F}} \cdot p\mathrm{H}.$$

These facts can only mean, in our view, that hydrogen atoms can be transferred from the sulphydryl group to the electrode, but that the disulphide compound is quite incapable of taking hydrogen back again from the electrode. The system is, therefore, essentially an irreversible one.

The behaviour of the disulphide group is interesting. It seems to require a certain critical *r*H value before it can be reduced at all. For instance, the potential measurements show that it is not reduced by the electrode at, say, *r*H 13 (for glutathione), but if the *r*H of the electrode is lowered (*i.e.*, the hydrogen pressure increased) by applying a negative potential to the electrode from an external source a point is reached where it is reduced. In other words, the disulphide group can be reduced electrolytically. Again, zinc dust suspended in neutral solution has an *r*H small enough to reduce indigo carmine—that is to say, an *r*H less than 8, but it is quite unable to reduce the disulphide group. If, however, the *r*H is made still smaller, by increasing the hydrogen ion concentration of the solution, the disulphide compound becomes reduced.

To return to the sulphydryl system, however, it is clear that since the process is irreversible it cannot be in equilibrium, and must be treated kinetically. If the hydrogen were transferred irreversibly to the electrode, and there were no compensating process, we should expect the hydrogen pressure to rise steadily to very high values. The potential measurements show, however, that this is not the case, and it is therefore clear that we must look for some compensating process tending to remove hydrogen from the electrode. The most likely one would seem to be the diffusion of hydrogen gas away from the electrode. This, as has been pointed out above, is bound to occur in fact, but it will not appreciably affect the potential given by those systems, such as those we have considered, which react very rapidly with the electrode. It may, however, well have an effect in this system. The —SH group is, to judge from experiments on rates of reduction, peculiar in being a very much more slowly reacting group than those substances which form the reversible systems of the type studied by Clark, so that it is possible that diffusion may not be slow in comparison with the transfer of hydrogen to the electrode.

Now assuming that this is the actual compensating process, it is clear that, when the rate of supply of hydrogen by the sulphydryl compound is equal to the rate at which hydrogen gas leaves the electrode, a steady state will be reached, and a constant potential will be obtained. The system will automatically adjust itself to this steady state, as the surface concentration of hydrogen atoms will increase or decrease until it is attained.

The process supplying hydrogen to the electrode may be represented thus—

$$2\mathrm{R.SH} \longrightarrow \mathrm{R.S.S.R} + 2\mathrm{H},$$

and its velocity will therefore be proportional to $[\mathrm{RSH}]^2$. The slowest part of the compensating process will probably be the reaction

$$2\mathrm{H} \longrightarrow \mathrm{H}_2$$

in accordance with what would appear to be the most satisfactory theory of the phenomena of over-voltage. The rate of the compensating process will, therefore, be proportional to $[\mathrm{H}]^2$. Now, when the steady state is reached, these two rates will be equal, so that

$$[\mathrm{R.SH}]^2 = k'[\mathrm{H}]^2.$$

We saw in the section on the hydrogen electrode that

$$p = k_3\,[\mathrm{H}]$$

so that substituting in the Nernst equation we get

$$\mathrm{E} = \frac{\mathrm{RT}}{\mathrm{F}} \log \frac{[\mathrm{H}^{\cdot}]}{k_3[\mathrm{H}]} \quad \text{and} \quad \mathrm{E} = \frac{\mathrm{RT}}{\mathrm{F}} \log \frac{[\mathrm{H}^{\cdot}]}{k''[\mathrm{R.SH}]},$$

which may be re-written

$$\mathrm{E}_h = \mathrm{E}_0 - \frac{\mathrm{RT}}{\mathrm{F}} \log\,[\mathrm{R.SH}] - \frac{\mathrm{RT}}{\mathrm{F}} \cdot p\mathrm{H},$$

which is the experimental equation previously given for this system.

It may be thought that in deriving this equation a thermodynamic equation has been applied to a system which is not in equilibrium, but this is not so. The surface concentration of hydrogen, [H], which is determined by irreversible processes, was calculated from kinetic considerations; and the thermodynamic equation was applied only to the relation between the surface hydrogen atoms and the hydrogen ions in solution, which *is* an equilibrium.

If we take into account the fact that the — SH group ionises in alkaline solution we get, by the application of the methods illustrated previously, the modified equation

$$E_h = E_0 - \frac{RT}{F} \log S_r + \frac{RT}{2F} \log (K[H^\cdot] + [H^\cdot]^2$$

where K is the ionisation constant. This equation is in full agreement with the experimental results of Dixon and Quastel, but at present the data are insufficient for the accurate determination of K. For cysteine it appears to be, very roughly, 2×10^{-9}.

In such systems as the present one we must define carefully what is meant by *r*H. To have any useful physical meaning it must equal log 1/P, where P is the hydrogen-gas pressure which would be in *equilibrium* with the actual hydrogen-atom concentration [H]. It must not be defined with reference to P′, the *actual* pressure of hydrogen gas in the electrode, which will not be equal to P in such systems. The *r*H so defined, which is the value calculated from the potential, is then analogous to that of the other systems considered.

The view of this system put forward here indicates that the reduction potential (and *r*H) of — SH compounds, as measured by, *e.g.*, a gold electrode, is not a measure of their true reducing power, since if the compensating process could be stopped much higher hydrogen concentrations, and therefore smaller *r*H values, would be produced. A number of facts confirm this. For instance, measurements with a gold electrode indicate that a N/500 solution of reduced glutathione has an apparent *r*H of about 13. I have found, on the other hand, that such a solution is capable of reducing indigo carmine to completion, so that its true *r*H, if one may use the phrase, must be smaller than 8. The same applies to certain other dyes.

Further, if our view is correct, we should get a much higher hydrogen concentration, and therefore a more negative potential and a lower *r*H, if we used an electrode of a metal in which the rate of the compensating process is very small; in other words, if we used a metal with a high hydrogen over-voltage. I have been able (in conjunction with Dr. Kodama) to verify this prediction experimentally. Mercury has a high hydrogen over-voltage, and does not react chemically with the solution, so that one would predict that a mercury electrode would be negative to a gold electrode in a solution of a sulphydryl compound. Measurements were made of the difference of potential between gold and mercury electrodes in the same solution of reduced glutathione. The solution was kept stirred by a stream of nitrogen, which also removed any traces of oxygen. I found that the mercury was about 200 millivolts negative to the gold—a very large difference. The potential readings were remarkably steady and constant, and to judge from only a few experiments the mercury electrode behaved in a similar manner to the gold when the *p*H and — SH concentrations were varied. When quinhydrone solution was placed in the vessel instead of the glutathione solution the same mercury and gold electrodes gave no difference of potential. The *r*H of the glutathione solution calculated from the potential given by the mercury electrode was about 7, which is in agreement with the indigo experiment mentioned above. The disulphide form was found not to affect the potential given by the mercury electrode, so that the critical *r*H for its reduction must be below 7.

The above facts afford good support to the view of the sulphydryl system put forward in this section. A satisfactory explanation has hitherto been lacking.

Summary.

1. It is shown that the phenomena of oxidation-reduction potential can be predicted from the work of Wieland, and a theory in accordance with Wieland's theory of oxidation is developed and extended in various directions.

2. An explanation of the anomalous behaviour of the sulphydryl system is suggested.

The writer wishes to express his sincere thanks to Prof. Sir F. G. Hopkins for his interest and encouragement.

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On the Occurrence of the Œstrous Cycle after X-Ray Sterilisation Part II.—Irradiation at or before Birth.

By A. S. Parkes (Beit Memorial Research Fellow).

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(From the Department of Physiology and Biochemistry, University College, London.)

[Plate 6.]

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I. *Introduction.*

The first paper of this series dealt with the effects on the œstrous cycle of X-ray sterilisation of the young female mouse at 3 weeks old. The conclusions arrived at were briefly as follows :—

(1) Adequate exposure to X-rays causes disintegration of the Graafian follicles, and when puberty is subsequently reached the ovary contains no organised follicles, and in most cases no luteal or other tissue recognisable as being of follicular derivation.

(2) In spite of this elimination of the cyclic structures of the ovary, the accessory organs of the majority of the animals experienced the normal periodic changes which constitute the œstrous cycle.

(3) These results made it quite clear that neither Graafian follicles nor corpora lutea are necessarily essential for the periodic occurrence of œstrus, but no satisfactory explanation was arrived at of the means whereby the cyclic action of the œstrous-producing hormone is regulated.

Since these results were entirely opposed to what has usually been thought of the regulation of the œstrous cycle,* it seemed desirable that the same type of experiment should be repeated under somewhat different conditions.

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* A summary of current views of the mechanism of the œstrous cycle was given in Part I and will not be repeated here.

With this end in view, two series of animals were sterilised earlier than those previously dealt with, in the hope that early sterilisation would :—

(1) Prevent the formation of corpora lutea atretica;
(2) Show more clearly the effect of the elimination of the follicles on the development of the accessory organs and on the occurrence of the œstrous cycle; and
(3) Make possible greater histological changes in the irradiated ovary itself.

The present paper, therefore, is concerned with the sterilisation of mice fœtuses *in utero* and with sterilisation of the young immediately after birth in the first week of suckling.

The full details of the histological aspects of the work will be dealt with, in collaboration with Dr. Brambell and Miss Fielding, as a separate communication, and, since this present paper is solely concerned with the physiological side, only such reference will be made to the histology of the irradiated ovaries as is necessary for the discussion of the results.

As in the case of the work previously recorded in Part I, much is due to the advice and criticism of Prof. J. P. Hill, F.R.S., and Dr. F. H. A. Marshall, F.R.S., to whom my best thanks are due. Similarly, the irradiations have all been carried out by Mr. F. Melville, and this opportunity is taken of acknowledging my continued obligation to him.

During the time these experiments were being carried out the mouse colony was maintained with the aid of a grant from the Government Grants Committee of the Royal Society.

II. *Methods and Material.*

The mice used for these experiments were drawn from the same colony as those used for the first experiments. The general management of this colony has been described elsewhere (6), and since the experimental mice are kept in the same way, further description need not be given here.

Two separate series of mice will be described in this present paper. Before the work on the œstrous cycle was begun, pregnant mice were being irradiated with the object of ascertaining whether or not the sterilisation of fœtuses *in utero* was feasible, and if so, what results on the growth and development of the accessory organs of reproduction would follow this early destruction of Graafian follicles and spermatic tubules. When the question of the œstrous cycle arose, such females as were available from the young of this series were examined for the occurrence of œstrus. Considerable difficulty, however, was experienced in arriving at a dosage of X-rays which was sufficient to produce sterilisation without disturbing the pregnancy; and even when this had been done, it was found that the sterilising effects were very irregular. As a result of this it was decided to irradiate young immediately after birth, and to give a heavy dose which was known to produce sterility in young at 3 weeks old. This material irradiated at birth has been relied upon for most of the œstrous cycle work described in the present paper.

Identification of Animals.—As colony routine each series of experiments is denoted by one or more letters, the individual mice in each series being given a number in addition, and, since various series are involved in this paper, it may be of use to give the index letters of the series which will be discussed.

XVP.—Animals irradiated during the first half of pregnancy, *i.e.*, soon after the discovery of the vaginal plug.
FXVP.—Young of XVP. Only one litter was obtained.
SX.—Animals irradiated during the second half of pregnancy and afterwards used for lactation experiments, which will be described in a later paper.
FSX.—Young of SX.
PX.—Irradiated as SX, but differing in subsequent treatment.
FPX.—Young of PX.
BX.—Mice irradiated immediately after birth.

Dosage of X-Rays.—The technique of the irradiations has been fully described in Part I and need not be discussed again here. The smallest dose given to animals dealt with here was 10 minutes of the standard intensity described in Part I (5); this small exposure gave a barely perceptible colour change on Levi's pastilles. The largest exposures given up to the present have been of 80 minutes duration at the same intensity, an irradiation giving approximately ¼ B tint on Levi's pastilles. The latter dose has been found to produce sterilisation with regularity in young animals. Doses of only ⅛ B tint, however, have been found to produce sterility in some cases.

In the case of the BX series, the smallness of the young at birth quite precluded the possibility of affording protection to other than gonad regions of the body. In the irradiation of fœtuses protection, of course, is quite impracticable. This results in the whole organism being subjected to the irradiation. Many of the BX series became temporarily hairless at about 3 weeks old. Whether any other injurious effects are experienced is not yet known with certainty, but no noticeable lasting effects are produced.

Detection of Œstrus.—The detection of œstrus by the vaginal smear technique has been fully described by Allen (1) and the application to this experimental work has been dealt with in a previous paper on the cycle in normal animals (4) and also in Part I of this series (5). The normal mice in the colony were found to have an œstrous cycle of an average duration of 5·7 days when unmated. Sterile copulation, however, at an œstrous period adds about 5 days to the length of the next diœstrous interval; that is to say, where sterile copulation takes place the next œstrous period does not occur for an average of 11 days. Since the irradiated mice were for the most part mated with normal males, and since copulation was, of course, sterile, this distinction between the length of the cycle in the two cases is of importance.

As has been described in detail by various authors and also by the present writer, copulation can readily be detected up to 12 to 24 hours after by the presence in the vagina of the "vaginal plug," formed from the secretions of the seminal vesicles and prostate gland of the male. The detection of copulation in the sterilised female has been considered to be important from two points of view. In the first place, in animals such as mice which only copulate at the œstrous period, the act of copulation is the climax of œstrus and is the best criterion that an œstrous cycle exists. Secondly, the failure of copulation to result in pregnancy provided a useful initial guide as to whether or not sterilisation had been effected.

Detection of Pregnancy.—In the mouse no infallible signs of pregnancy are to be seen until about the tenth day, when blood (called by Long and Evans the "placental sign") appears in the vagina. At this time, also, careful palpation of the abdomen will in nearly all cases reveal the presence of fœtuses if pregnancy is established. Previous to the tenth day, however, it is impossible to detect pregnancy with certainty in the intact animal, and for experimental work during the early stages of pregnancy it is necessary to make the assumption that, with both sexes normal, the detection of the vaginal plug will probably be followed by pregnancy. Extensive controls on normal animals (6) have shown that about 80 per cent. of the females bear a litter to full time following the discovery of the vaginal plug. If this percentage is materially lowered in a significant number of animals, it may confidently be assumed that the normal course of early gestation is being disturbed.

III. *Experimental Records.*

In order to confirm in as definite a manner as possible the results obtained on animals sterilised at weaning time (3 weeks old), efforts were made to effect sterilisation at the earliest possible time. Experiments had already been made as to the effect of irradiation during the latter half of pregnancy, and it had been found that exposure at this time to doses sufficient in some cases to produce sterility did not affect the course of the gestation.

Irradiation in the First Half of Pregnancy.—Experiments were therefore made on the effects of irradiation at early stages of gestation. For this purpose thirteen females, XVP 1–13, were irradiated for 40 minutes (found by experience to be the least dose sufficient to sterilise) 4–6 days after the discovery of the vaginal plug. Of these the last three were dissected at various dates before parturition was due, and none showed any signs of pregnancy. Of the other ten, of which at least seven or eight should have produced normal litters on the probability basis, only two did produce litters, so that it must be assumed that the irradiation had in most cases so far disturbed the embryos as to make the continuance of gestation impossible. Of the two litters born, one litter of five (FXVP 2) all died within two days and are of no further interest, as post-mortem decomposition was too far advanced to make the gonads of any histological value. The other litter produced (FXVP 5) comprised one male and six females. These, though very poor at birth, were all reared to weaning time, and two to puberty. These two, both females, were mated with normal males, and were found to be quite fertile, so that the irradiation during early pregnancy did not appear to have been effective in sterilising the embryos. This conclusion was confirmed by the histological appearance of the gonads of the other young of this litter. This result is possibly due to the fact that the irradiation took place before the differentiation of the gonads from the peritoneum. Why the irradiation should destroy the embryos and lead to the termination of the gestation in the majority of cases is not apparent. It can, however, scarcely be due to any influence on the maternal ovaries. Only destruction of the corpora lutea would be likely to have this effect, and these bodies, even in those cases where the maternal ovaries were affected, followed the usual course of immunity to X-rays. The data relating to these irradiations during early pregnancy are summed up in Table I.

In view of these results of fœtal irradiation during early pregnancy, these experiments were abandoned from the point of view of the production of sterile young.

Irradiation during the Last Week of Pregnancy.—The irradiation of pregnant females in the last week of gestation was among the earliest of the X-ray experiments to be carried out, and since it was possible to form but little idea of what

Table I.—Effect of Irradiation during Early Pregnancy.

Animal.	Time of irradiation. Days after copulation.	Result.	Subsequent history of mother.*
XVP 1	6	No litter	Fertile when mated again.
XVP 2	6	Litter of 5 very poor young. All died	Fertile when mated again.
XVP 3	5	No litter	No copulation observed when mated again.
XVP 4	4	No litter	Copulated, but was infertile when mated again. Histological absence of follicles.
XVP 5	4	Litter of 7 (1 ♂ : 6 ♀). Young not sterilised	Copulated, but failed to become pregnant.
XVP 6	5	Abortion at 14 days	Copulated and became pregnant.
XVP 7	5	No signs of pregnancy	Copulated 5 days after irradiation and became pregnant. Copulated regularly afterwards, but failed to become pregnant.
XVP 8	5	Abortion at 15th day	Copulated and became pregnant on one occasion.
XVP 9	5	No signs of pregnancy	Fertile copulation took place.
XVP 10	5	No signs of pregnancy	Infertile copulation.
XVP 11	5	Killed 1 day after irradiation. Nodules in uterus	—
XVP 12	5	Killed 3 days after irradiation. Uterus full of fluid.	—
XVP 13	5	No signs of pregnancy when killed 5 days later	—

dose would be required to sterilise fœtuses by irradiating the pregnant mother, the first experiment consisted in giving three does 1 standard dose, *i.e.*, 10 minutes exposure of the intensity detailed elsewhere. The first of these does (PX 1) produced a litter of nine 10 days after irradiation, and the sex of seven of these was ascertained. The mother subsequently produced two further litters while under observation and was herself not sterilised; a result confirmed by histological examination of the ovaries, which, while showing more atretic follicles and oöcytes than are normally found, also possessed many normal follicles. Seven of the young of the litter irradiated during fœtal life (FPX 1 ♂ *a* and *b*, and FPX 1 ♀ *a–c*) were reared, and these were mated with each other in two batches, the first having one male and two females, and the second one male and three females. Four out of the five females produced litters within a month of being mated, 24 young in all being produced. These young were killed immediately and the females were all mated again with the males. One month later the whole seven were dissected.

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* These animals will be further discussed in a later paper.

All five females were found to be in various stages of pregnancy, 34 fœtuses in all being present; 58 fœtuses therefore were produced in nine litters by the first batch of mice irradiated during fœtal life, giving an average per pregnancy of 6·2. Since the average size of litter for normal mice in the same colony bred under the same conditions is 6·3, the dosage employed cannot be said to have produced any definite results in the direction of sterility. This empirical finding was confirmed by histological examination of the gonads of the irradiated animals.

The second of the irradiated pregnant females (PX 2) produced a litter of nine, 5 days after exposure. Of these, however, four were weakly and died during the first week. After parturition the doe herself was re-mated and subsequently produced another litter. She was then killed for dissection and the ovaries sectioned. Much the same condition was found as in the case of PX 1, *i.e.*, normal follicles and corpora lutea, but an excessive number of atretic follicles. The five remaining mice of the litter irradiated during fœtal life—one buck and four does—were reared and mated with each other at 9 weeks old. Although they remained mated for 2 months, no litters were produced, and when dissected at the end of this period no signs of pregnancy were observed in the females. The histological appearance of the gonads, however, failed to confirm the supposition that sterility had been produced.

The third of the females (PX 3) irradiated during pregnancy produced a litter on the day following irradiation. This litter, however, was extremely weakly and two of the five were either born dead or else died immediately after birth. The remaining three died in the course of 5 days. It was first thought that this high mortality might be connected with the irradiation, but later experience showed that much higher dosage than was originally used is required to influence seriously the vitality of fœtuses. The mother was mated again after the birth of the irradiated litter, but failed to breed. This failure, however, as shown by subsequent dissection, was not due to the obliteration of the follicular tissue of the ovaries.

It was obvious from these experiments that the comparatively small dosage employed was not nearly sufficient to sterilise fœtuses *in utero*. Soon after this five pregnant females (numbered SX 1–5) were X-rayed for an entirely different experiment, but the young were reared and examined from the point of view of the present investigation. Of these five sets of fœtuses two received 10 minutes irradiation of the standard intensity (the same as FPX 1–4), two 20 minutes, and one litter 30 minutes. These litters irradiated *in utero* showed the following results when the adult age was reached:—Female SX 4 was exposed for 10 minutes and gave birth to a litter of six 2 days later. Of these four, the two females were reared and were mated with normal males at 11 weeks old. Both animals, however, subsequently became sterile.

The next pregnant female (SX 5) was exposed for 10 minutes when 15 days pregnant, and produced a litter of seven at the normal time. Of these only three, one male and two females, were reared. These were mated to normal adults at 11 weeks old, and all three proved fertile. The histological findings in the case of the females showed that the ovaries had suffered no appreciable disturbance, follicles and corpora lutea being abundant in both. The uteri, including the muscular layers, were perfectly normal, and no abnormal corrugation of the uterine mucosa was observable.

Female SX 1 was irradiated for 10 minutes when 16 days pregnant, and again for a further 10 minutes 2 days later. On the following day this animal gave birth to a litter of five, of which three, one male and two females, were reared. The male died when 2 months old. The two females (FSX 1 ♀ *a* and *b*) were mated with normal males when 12 weeks old, and as pregnancy did not ensue were dissected a month later. Post-mortem examination of the first female revealed a non-gravid but apparently normal uterus, and large ovaries which appeared to contain corpora lutea. The second female also was not pregnant, but in this case the uterus was not well developed. The ovaries were large, but no corpora lutea were visible.

Female SX 2 was given exposures on the 15th and 17th days of pregnancy. A litter of four was produced at the normal time. Of these only one female was reared, and this was mated with a normal male at 11 weeks.

The last of the five pregnant females under discussion (SX 3) received 10 minutes exposure on each of 3 days (10th, 14th, 17th day of pregnancy). Parturition occurred at the normal time, but the litter was weakly, and only two were reared. Both of these, one male and one female, were mated with normals at 10 weeks old and proved to be quite sterile. Subsequent dissection suggested that complete inhibition of gametogenesis had been produced. The testes of the male were small, about one-sixth the volume of the normal testis, and were brown in colour as contrasted with the whitish of the normal. Histological examination confirmed this supposition, and further description of this animal (FSX 3 ♂) will be left to the next section of this paper. Dissection of the female revealed a similar state of affairs. The right ovary was very small, while the left was practically non-existent, and was only found after prolonged search among the fat body surrounding the ovarian capsule. The uterus was well developed. Microscopical examination of the ovaries showed that one ovary (the left) was completely devoid of follicles, but the right ovary possessed one very large follicle. No special meaning need be attached to the abnormal size of this follicle however; the fact that excellent nutrition would be available for a single surviving follicle supplies all the required explanation. Further discussion of these ovaries will be reserved for the histological paper.

In view of these results, two further pregnant does were subjected to longer exposures.

The first of these two does (PX 5) was irradiated for 40 minutes, 3 days before giving birth to a litter of five. Of this litter one was a male and four were females, but one of the latter (FPX 5 ♀ *a*) died when 5 weeks old. When 9 weeks old the second female of the litter (FPX 5 ♀ *b*) was mated, and vaginal smears were begun at the same time. The first copulation was noted 3 weeks later, but as the animal died 3 days later it was not possible to ascertain whether or not pregnancy had resulted. Dissection showed clearly that the ovaries were not sterilised, and, although accurate histological examination was precluded by the post-mortem degeneration which had occurred, both follicles and corpora lutea could be discerned in the sections. The remaining females of the litter (FSX 5 ♀ *c* and *d*), which had not grown well, were mated at 12 weeks old. Both copulated within 4 days, and as the vaginal smears became suspicious, both were dissected 10 days later. In both cases one ovary appeared to be sterile, while the other contained obvious corpora lutea, and each animal had conceived in the cornu corresponding to the fertile ovary. Histological examination showed that although the left ovary of ♀ *c* and the right ovary of ♀ *d* had been sterilised, the other ovary in each case appeared to be quite normal. PX 6 was irradiated for 40 minutes also, and produced a litter of one female and four males 3 days later. The animal (FPX 6 ♀ *a*) was mated when 10 weeks old and proved to be quite sterile, in spite of the fact that four males in all were tried. Since histological examination confirmed the diagnosis of sterility, discussion of this animal will be left to the next two sections.

The effects of fœtal irradiation are, therefore, very varied, but it must be noted that difference in position in the maternal pelvis and the exact curvature of the gravid uterus must result in a very considerable variation in the amount of protection afforded by the mother to individual fœtuses, and even in the amount afforded by the fœtuses to each other. The results, therefore, are not so unreasonable as they might superficially appear. At the same time, however, the uncertainty is disconcerting from an experimental point of view, and it was decided that irradiation of the new-born young would be much more satisfactory.

Irradiation at Birth.—For the purpose of sterilising the young at birth, two irradiations of 40 minutes each, or about $\frac{1}{4}$ B tint on Levi's pastilles, were given, thus bringing this series into line with the M series described in Part I. The usual plan was to give the first irradiation as soon as practicable after birth, and to make the second exposure during the ensuing week. Twenty litters, comprising 94 (BX 1–94) animals, have in all been irradiated at this time. Of these 94 animals, 46* were males and 48 females. The apparently small size of litter is accounted for by the fact that, in order to give the irradiated young a better chance, the larger litters were reduced in size. In spite of these precautions, however, the irradiations, as mentioned above, seemed to have a general temporary effect on the whole organism, and as a result of this many of the young were subsequently undersized, and showed temporary abnormalities, such as hairlessness. The mortality during rearing, particularly from diarrhœa, was, therefore, unusually high. Of the 48 females, 6 (BX 33, 34, 35, 37, 42, 44) were killed at graduated intervals after irradiation for the purpose of studying the immediate histological changes. Of the remaining 42, only 25 were reared to puberty and examined for the occurrence of the œstrous cycle, the other 17 dying at various stages of development. Those dying before puberty appeared to include those most affected generally by the irradiation, and the 25 which survived were for the most part in fair condition. Of the 25, 1 (BX 74) died suddenly, and the ovaries were not obtained in a fit condition for histological examination, while BX 88 still retained one small follicle and therefore is not included in the sterilised series. The animal is, however, of interest owing to the fact that a good deal of cornified matter was observed during the whole 15 days which elapsed between the two œstrous periods observed.

The œstrous histories of 23 females sterilised immediately after birth are therefore available, and these will be discussed in Section V of the paper.

IV. *Histology of the Ovary irradiated before or at Birth.*

The FPX and FSX material is so limited that it does not furnish much evidence as to the changes taking place after irradiation by itself. By comparison, however, the changes seem similar to those occurring in the ovaries of animals X-rayed at birth. The majority of the oöcytes in both groups seem to disappear completely within the first few days after irradiation. Some, however, grow and acquire a follicle. These follicles may persist for a considerable time, but ultimately degenerate completely, become cystic, or form corpora lutea atretica, which may persist for an indefinite period. While these changes are proceeding, the germinal epithelium appears to proliferate finger-shaped cords of epithelial cells which replace the pre-irradiation tissues of the ovary.

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* The subsequent history of these males will be dealt with in another paper.

Histologically the ovaries of the adult BX's broadly resemble those of the M's, and all the structures described in the latter are found in the former in varying degrees of development. Cords of the second post-irradiation proliferation are only found in 11 of the 24 animals examined (77, 62, 64, 24, 51, 15, 56, 76, 46, 63, 75), while they are poorly developed in the first eight of these. All the animals completely lack follicles, with the exception of BX 88, which had one small one. This animal and BX 77, 32, 16, 56, 76 and 70 had several corpora lutea atretica. Some of these are retrogressing, others are apparently healthy, but, as will be shown, none appears to have any effect on the œstrous cycle. Many of the ovaries contain cysts, probably formed from former follicles. Most of the ovaries exhibit the anomalous epithelial canals described in M 15, 16 and 42. The major part of all the ovaries is composed of cords of the first post-irradiation proliferation, and the animals can conveniently be divided into three groups according to the condition of this tissue.

BX 16, 32, 62, 77, 88, 90 and 91 comprises the first group, which is characterised by the vacuolated nature of the cells of the cords of the first proliferation. These cells have not become much enlarged after their separation from the epithelium, but are very vacuolated, and somewhat shrunken and unhealthy-looking in section. BX 77 is the most striking example.

BX 15, 46, 56, 63, 64, 75, 76 and 89 comprise the second group, in which the cells of the first proliferation have enlarged somewhat and become more glandular in character. In some they have begun to resemble luteal cells to a limited extent. These ovaries are therefore similar in these respects to the large majority of the M series.

BX 13, 14, 24, 25, 31, 50, 51, 69, 70 form the third group and are characterised by the remarkable resemblance of the tissue of the first proliferation to corpus luteum. This is so striking that the cells themselves are often indistinguishable from luteal cells, although the arrangement in definite cords, not spherical bodies, readily distinguishes them. BX 69 and 70 differ from the others in that the cells of this tissue are much vacuolated, probably by fat infiltration, though large and healthy-looking.

A full description of the histology of these ovaries belongs to another paper, but the animals will be grouped as above for the purpose of discussing the œstrous cycle histories.

V. *The Œstrous Cycle after Sterilisation.*

Irradiation before Birth.—Only one animal is available for discussion in this category. Of the females irradiated before birth and subsequently examined for the occurrence of œstrus, only FPX 6 ♀ *a* was found to be completely sterilised. The pregnant mother received an $\frac{1}{8}$ B tint irradiation 1 day before parturition and vaginal examinations were commenced when FPX 6*a* was 10 weeks old. Pronounced cornification was found immediately and this continued for some days, a condition which, as noted elsewhere, is often found in the young normal female. Daily examination was continued for 27 days, and during this time three periods of œstrus each attended by copulation were observed. The two complete cycles thus observed had a duration, however, of only 6 days and 7 days respectively. Since this time corresponds much more closely to the length of the cycle in the unmated animal than to the length of the cycle after sterile copulation, it would seem that the mechanism whereby copulation postpones the next œstrus had become deranged. This animal was killed immediately after the discovery of the third copulation. Dissection revealed a typical œstrous condition of the uterus, and microscopical examination of the contents of the cornu showed that great numbers of spermatozoa were present.

The ovaries contained neither corpora lutea nor Graafian follicles.

Irradiation at Birth.—Twenty-three females are available for discussion here, and to save repetition in the histological details it will be convenient to classify the animals into groups according to the salient features of the ovary. It has been pointed out above that many of the ovaries of these animals sterilised at birth showed a condition almost identical with the ovary of the typical mouse irradiated at 3 weeks old (Group 2). At the same time certain of the BX series possessed ovaries which appeared to be almost entirely composed of luteal-like tissue (Group 3). The few animals with markedly parenchymatous ovaries form a further convenient group (Group 1).

The œstrous histories of the first group of animals (as classified by the ovarian types given in Section IV) are as follows :—

BX 16.—The initial growth of this female was poor and 18 weeks elapsed after the first irradiation before vaginal examination was begun. Observations were made for 89 days, the animal being mated during the whole of this time. During the 89 days, nine periods of œstrus were observed, and of these, eight were accompanied by copulation. The lengths in days of the eight complete cycles were respectively *47**–*6–10–8–6–3–3*–6, the last being unpreceded by copulation. The average length of the cycles accompanied by copulation is thus 12·3 days, which compares very closely with that of 11·0 found for normal mated mice (Parkes (4)). The significance of this similarity is, however, exaggerated in so far as it fails to take into account the variation in the lengths of the individual cycles. This variation is far greater than that normally found in the intact animal, and suggests some disturbance of the regulatory mechanism. The animal was killed immediately after the detection of the last vaginal plug, and dissection revealed a typical œstrous condition of the uterus (Plate 6, figs. 1 and 3). In spite of the complete absence of follicles, an apparently luteal body was found in one ovary, but, as pointed out in Section IV, this does not necessarily mean that any follicle reached maturity or that any follicle had been present during the time when the vaginal examinations were being made. In addition, the continued sterility of the animal after repeated copulation with a normal male strongly suggests that no ovulation took place. Since the second ovary contained neither follicles nor luteal tissue, this case affords further evidence that destruction at an early age of all Graafian follicles is not incompatible with the subsequent occurrence of numerous, if irregular, periods of œstrus.

BX 32.—This animal was examined for 84 days in all, and was mated during the whole time. Two periods of œstrus were observed, but the cycle was distinctly peculiar. Thirteen days initial diœstrus was followed by 21 days intense cornification without copulation. The next 23 days were occupied by normal diœstrous smears, while during the next 3 days cornification again occurred. No copulation, however, took place. After this a 4 day diœstrus was observed, and throughout the remaining 20 days during which examinations were made pronounced cornification was again apparent.

BX 62 was examined for 14 days only before death from diarrhœa at 13 weeks old. The female was mated with a normal male on the day before vaginal examinations were started, and on the first day of observation copulation was found to have taken place. In spite of this, however, cornified cells only were found in the vagina on the following day, and on each of the remaining 12 days on which examinations took place the same condition only was found. No further copulation, however, was observed. This peculiarly persistent cornification is clearly comparable with that observed in BX 32.

BX 77.—Vaginal examinations were begun on this animal 67 days after birth and were continued for 20 days. The animal was not mated. Cornification became apparent 3 days after the examinations were begun and continued for 4 days. No further signs of œstrous activity were observed.

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* In these "cycle formulæ" figures for cycles preceded by copulation are printed in italics.

BX 90.—This animal was examined at 14 weeks old for 22 days. In this case, however, mating with a normal male was made. One œstrous period attended by copulation after 1 day of cornification was noted. Autopsy was performed 18 days after this time. The broad ligament and mesenteries were not abnormally fat, and the uterus appeared to be well developed. This was confirmed by histological examination.

BX 91.—This animal had a similar history to the preceding one. One œstrous period, attended by 2 days' cornification, was observed. The mesenteries and broad ligament were found on autopsy to be rather fatter than is normally the case, but not to such a marked extent as those to be described later.

From these five cases it is clear that the production of a parenchymatous type of ovary as the result of irradiation at birth is quite compatible with the appearance of œstrous symptoms. At the same time in two out of the three cases recorded above the parenchymatous ovary is associated with a marked exaggeration of the cornification stages of the cycle. The nature of the correlation, if any, is at present obscure.

The eight animals belonging to the second ovarian type all experienced at least one œstrous period. The details are as follows :—

BX 15.—Smearing was started on this animal when 13 weeks old, and was continued for 15 days before death occurred. During this time two œstrous periods were observed. The animal was mated, but only the second of the periods of œstrus observed was attended by copulation. Four days of cornification were observed at the first period, and 2 days of cornification preceded the discovery of the vaginal plug at the second. The time elapsing between the termination of the first and second œstrous periods was 5 days, which is a perfectly normal length for a cycle not preceded by copulation.

BX 46.—Beginning when the animal was a little over 10 weeks old, examinations were made for 104 days, the animal being kept mated. During this time 13 periods of œstrus, embracing 12 complete cycles, were observed. Of these periods five were attended by copulation. The lengths of the cycles were as follows :—*11*–8–*7*–5–5–4–*5*–8–8–*7*–28–*4*. Normal features were observed in the majority of these cycles. The 11th cycle was, however, peculiar in consisting of 4 days' diœstrus and 23 days' cornification, after which copulation took place. The exact cause of this aberration was not apparent. This abnormally long cycle raises the average length of cycles unpreceded by copulation to 9·4 days, as against an average of 6·8 days for cycles preceded by copulation. Excluding the aberrant cycle, however, the average for the former is 6·3 days. In any case it is clear that the radical difference which is normally found between the length of the cycle in the unmated animal and the length after sterile copulation is not observable in this animal, and it seems possible that irradiation interferes with the œstrus postponing mechanism which is set in motion by copulation. It will also be noticed that, in common with BX 16, the variation in the length of the cycles is very marked, and the same caution with regard to the inadvisability of attaching any great importance to averages is equally applicable. The animal was killed 4 days after the discovery of the last vaginal plug. The accessory organs appeared to be quite normal, and histological examination of the uterus revealed a normal diœstrous condition.

BX 56.—This animal showed remarkably good growth, and it was found possible to mate it with a normal male at a little over 7 weeks old. Vaginal examinations, which were started at the same time, were made for 104 days. Five œstrous periods were observed in all, and copulation took place in each instance. The four cycles observed were respectively *21–19–24–26* days in length, and had an average duration of 22·5 days. This is considerably above the normal length, even after sterile copulation, but the regularity is striking. When killed, the accessory organs were found to be quite normal, the uterus having a perfectly typical diœstrous condition.

BX 63.—Vaginal examinations were begun on this animal a little over 11 weeks after birth, but owing to a bad attack of diarrhœa it was found necessary to kill the animal 13 days later. During this time, however, two periods of œstrus, both accompanied by copulation, were observed. The one complete cycle detected extended over 9 days. The post-mortem examination took place 2 days after the discovery of the second vaginal plug, which, as in the case of the first one, was abnormally retained in the vagina for two days. On microscopical examination the uterus was found to be small, but otherwise to present a typical diœstrous condition.

BX 64.—As in the case of the last animal this was a little over 11 weeks old when vaginal examinations were begun. During the 23 days on which smears were made, four œstrous periods were observed. Although constantly mated, however, copulation only took place at the last of these periods. The three cycles had a duration of 5–3–10 days respectively, the average of 6 days being quite normal for cycles unpreceded by copulation. The animal was killed 1 day after the detection of the vaginal plug which was found at the last œstrous period. Dissection showed a perfectly normal diœstrous uterus.

BX 75.—This animal showed bad growth and was nearly 14 weeks old before it was possible to start vaginal examinations. These were made for 58 days and three periods of œstrus were observed. The animal was unmated for the first 42 days, but afterwards mating with a normal male was made. As a result of this, the last œstrous period terminated in copulation. The œstrous history of this animal is somewhat strange. The first four daily examinations revealed exclusively cornified cells. Following this a 4 days' diœstrus was superseded by 3 days during which cornified cells only were found. No further sign of œstrous activity was noticed until the 49th day of observation, when cornified cells again appeared. Eight consecutive days of purely cornified smears terminated in copulation. The two cycles, therefore, have the very unequal lengths of 7 days and 47 days, neither being preceded by copulation. Autopsy took place immediately after copulation terminated the last œstrous period. The uterus was macro- and micro-scopically in a typical condition of œstrus (Plate 6, figs. 2 and 4). The vagina, too, showed the typical œstrous condition, *i.e.*, cornification of the epithelium.

BX 76.—Observations were begun on this animal 65 days after irradiation, and were continued for 67 days. During this time five periods of œstrus (unattended by copulation, since the animal was unmated) were observed. The intervals were, however, most irregular, the actual lengths of the cycles in days being :—44–3–3–3. The cornification found at each of the five œstrous periods was, however, quite normal and lasted for 3–2–1–1–1 days respectively. Autopsy was made 3 days after the last œstrous period, and the uterus was found to be in a typical diœstrous state. The mesenteries were rather fat, but to nothing like the degree found in other cases (see later). Histological examination showed nothing abnormal in the accessory organs.

BX 89.—Observations were begun on this animal when it was nearly 14 weeks old, and were continued for 22 days. During this time four periods of œstrus were observed. The animal was not mated. The three complete cycles were respectively 6–6–4 days in length, which is quite normal for the cycle unpreceded by copulation. The animal was killed 3 days after the end of the last œstrous period. The mesenteries were very fat and the uterus macroscopically looked normal but small. Microscopically, the uterus was normal except that the stroma appeared to be somewhat under-developed.

Animals of the first and second classes, *i.e.*, animals with ovaries comparable to those of the majority of the M series, have now been described. Since the conditions found in both of these two groups are not incompatible with the occurrence of an œstrous cycle, the œstrous histories of the animals are summarised in the following table :—

Table II.

No. of animal.	Days observed.	Œstrous periods.	Length of cycles.	Average length.	
				Preceded by copulation.	Unpreceded by copulation.
BX 16	89	9	47–6–10–8– 6–3–3–6	11·9 (7)	6 (1)
BX 32	84	2	26	—	26 (1)
BX 62	14	1	—	—	—
BX 77	20	1	—	—	—
BX 90	22	1	—	—	—
BX 91	22	1	—	—	—
BX 15	15	2	5	—	5 (1)
BX 46	104	13	11–8–7–5– 5–4–5–8– 8–7–28–4	6·8 (5)	9·4 (7)
BX 56	104	5	21–19–24–26	22·5 (4)	—
BX 63	13	2	9	9 (1)	—
BX 64	23	4	5–3–10	—	6 (3)
BX 75	58	3	7–46	—	26·5 (2)
BX 76	65	5	44–3–3–3	—	14 (4)
BX 89	22	4	6–6–4	—	5·3 (3)

From this table the following values may be calculated :—

Total œstrous periods in classes 1–2	53.
Proportion accompanied by copulation in mated animals..	56 per cent.
Average length of 17 cycles preceded by copulation ..	12·7 days.
Average length of 22 cycles unpreceded by copulation ..	11·1 days.

As regards these figures, however, it must be remembered that the actual average may be unduly influenced by one or two markedly eccentric cases. Thus the average of 11·1 days for the 22 cycles unpreceded by copulation is largely due to the abnormal cycles of 28, 46, 44 and 26 days found respectively in BX 46, 75, 76, 32. The remaining 18 cycles have an average length of only 6·3 days, which is entirely normal.

The third group of animals, as classified by ovarian character, were distinguished in most cases by the non-occurrence of œstrus. In the remaining cases the œstrous cycle had appeared only for a short time, and considerably before autopsy. The individual histories of these animals are not sufficiently important to be considered separately, and Table III summarises the cases.

Table III.

No. of animals.	Time observed.	Œstrous periods.	Length of cycle.	Time of last œstrus before autopsy.	Condition of uterus.	Condition of mesenteries and broad ligament.
	Days.			Days.		
BX 13	35	0	—	—	Infantile ...	Loaded with fat.
BX 14	35	0	—	—	,, ...	,,
BX 24	37	2	6	27	Fairly well developed	—
BX 25	35	0	—	—	Ditto	—
BX 31	35	0	—	—	Infantile	Loaded with fat.
BX 50	70	1	—	69	Fairly normal	Grossly fat.
BX 51	36	0	—	—	,,	
BX 69	66	1	—	64	Poorly developed One side more so than other	Viscera very fat.
BX 70	66	2	11	47	Fairly normal	,,

Relative to this table the following remarks may be made:—

(1) Of the nine animals possessing ovaries largely composed of luteal-like tissue, four only were found to experience even one period of œstrus.

(2) Of the four experiencing œstrus, two had only one period and two had two periods. In the latter two only, therefore, was a complete cycle observed. In no case was an œstrous period observed later than 27 days before autopsy.

(3) Since it is clear that destruction of the follicles alone is not sufficient to stop the œstrous cycle, it is necessary to suppose that the unusual development of this mass of luteal-like tissue which characterises the ovaries is the cause of the absence of the cycle in these animals.

(4) In four of the nine cases this aberration of the periodic function of the ovary was associated with a complete infantile condition of the uterus.

(5) The mesenteries of five of the nine animals were found to be grossly fat.

The following is a summary (Table IV) of the three groups of the animals sterilised immediately after birth and of the one animal sterilised before birth :—

Table IV.

	Irradiated at birth.		Irradiated before birth.	Total.
	Groups 1 and 2.	Group 3.		
Ovarian character	Little or no luteal-like tissue.	Markedly luteal-like.	Slightly luteal only.	
No. of animals	14	9	1	24
No. not having œstrus	0	5	0	5
Percentage not having œstrus	—	55·6	—	24
Total œstrous periods observed	53	6	4	63
No. of complete cycles	39	2	3	44
Cycles per animal	2·8	—	3	—

VI. *Discussion.*

The experimental work described above fully substantiates the result discussed in Part I of this series of papers, *i.e.*, that complete destruction of the Graafian follicles as organised units does not necessarily inhibit the occurrence of the œstrous cycle. In Part I, 53 periods of œstrus were recorded in 25 animals sterilised at 3 weeks old. In the present paper 63 periods of œstrus are recorded in 24 animals sterilised at or before birth. Of these, however, six only occurred in nine of the animals which were peculiar in having ovaries composed almost entirely of luteal-like tissue.

Possible criticisms of this type of work, and the steps taken to answer them in advance, were discussed in Part I. The most important were :—

(1) That ovarian tissue might have regenerated elsewhere, as may happen after ovariotomy, and that follicles in this regenerated tissue might account for the occurrence of the œstrous cycle.

(2) That the apparent cyclic activity of the reproductive organs of the sterilised mice did not constitute a process analogous to the normal œstrous cycle.

Since, however :—

(1) Ovariotomy of the sterilised gonad immediately and invariably stops the cycle, it is clear that the actual sterilised tissue removed is the actuating factor,

(2) Every feature of normal œstrus except ovulation occurs in the sterilised mouse,

these possible contentions have been adequately disposed of.

The Inhibitory Action of Luteal Tissue.—In the case of the present batch of animals, the issue is complicated by the fact that the nine animals of the third group were practically devoid of œstrous phenomena, and that this condition was associated with the presence of large amounts of luteal tissue in the ovaries. For reasons stated above it is necessary to suppose that this association is more than accidental. In the case of the animals (BX 24, 50, 69, 70) which showed one or two cycles early in their history, it is to be supposed that the development of the luteal tissue was somewhat later than in the cases in which no œstrous phenomena were observed. In this connection it is of significance to note that of the animals that never showed œstrous phenomena, only two had uteri even approaching normal development. Two views are possible as to why the presence of this luteal-like tissue, arising not from atretic follicles, but from the first post-irradiation ingrowth, should inhibit œstrus :—

(1) It has been concluded elsewhere that the œstrus-producing hormone is probably produced in these sterilised animals by the first post-irradiation ingrowth, and since in the case of the ovaries composed largely of luteal tissue this proliferation has changed its character entirely, it may be argued that "œstrin" is not being produced. This view is supported by the fact that the infantile nature of the uteri and the gross general fatness of the animal in some of the cases suggests most strongly that the general endocrine function of the ovary is disturbed by the invasion of luteal tissue.

On this view, therefore, the early conversion of practically the entire ovary to luteal-like tissue prevents both the development of the accessory organs and inhibits the occurrence of the œstrous cycle by preventing the elaboration of the requisite hormones. Where, however, the conversion to luteal tissue is later, the uterus develops and œstrous phenomena occur at the beginning of puberty, but subsequently the cycle stops and the uterus tends to atrophy.

(2) The other possible view of the action of the luteal tissue is to suppose that while the normal œstrous-producing stimulus of the ovary is present, the luteal tissue actually elaborates the œstrous-inhibiting substance which is produced by the normal corpus luteum. Colour is given to this hypothesis by the fact that the luteal tissue derived from the first post-irradiation proliferation is remarkably similar to normal corpus luteum and by the fact that it appears to be highly glandular and quite healthy. Contrary evidence, however, is not lacking. In the first place it is difficult to see why an œstrous-inhibiting substance should inhibit the normal development of the uterus and lead to the infantile organs found in three out of the five cases showing no œstrous period at all. In the second place, we have failed completely to show that any

permanent or even temporary œstrous-inhibiting action is brought about by the corpora lutea atretica formed in some cases from the degenerating follicles, and, furthermore, evidence will be brought forward in Part IV to show that the normal corpus luteum of pregnancy or lactation, when caused to become histologically persistent by the X-ray elimination of the follicles, fails to exhibit an œstrous-inhibiting action for a longer period than is normally the case, *i.e.*, about 3 weeks.

In the circumstances it seems most reasonable to accept the first view, and to suppose that the normal endocrine function of the sterilised ovary is entirely deranged by the intense proliferation of luteal tissue. In either case the dynamics of the ovary under these conditions are difficult to understand, but this aspect of the question will be dealt with more fully in conjunction with Dr. Brambell and Miss Fielding in considering the detailed histology of these ovaries.

It should be emphasised here, however, that this anœstrous condition found in conjunction with luteal-like ovaries is not primarily due to the destruction of the Graafian follicles, but is due to secondary changes in the sterilised ovary.

The Occurrence of Luteal Tissue in the Sterilised Animals already described.—In the M series of mice sterilised at weaning time recorded in Part I, entire absence of cycle or absence after one or two early periods was noted in a few cases. In one case (M 6 *e*) this was associated with a very extensive development of tubules in the ovary. In two other cases (M 7 *a* and *c*), however, it was associated with fatty degeneration. These two animals were observed to have one œstrous period each in the first month of examination, but during the succeeding 2 months no œstrous signs were observed. In view of the luteal-like nature of the ovaries, these two animals may very well be considered as analogous to Group 3 described above.

The Regulation of the Œstrous Cycle in the Sterilised Animals.—In Part I it was concluded that some functional periodicity was maintained by the sterilised ovary, but no histological signs of cyclic change could then be detected, and no such signs have since been detected in any series of sterilised ovaries. It is not impossible, however, though it seems decidedly improbable, that some cyclic histological change takes place in these ovaries. Certainly nothing analogous to the alternation of organised follicles and corpora lutea in the normal ovary is found. Since, however, it has been definitely shown that the sterilised ovary gives rise to the cyclic changes in the accessory organs, it is necessary to postulate some periodic functional activity of the sterilised ovary. This conception may now be developed a stage farther. Such periodic functional activity might arise from three mechanisms :—

(1) Periodic elaboration of the œstrous-producing hormone.

(2) Continued production of œstrin with the periodic production of an inhibiting substance such as is normally elaborated by the persistent corpus luteum.

(3) Continued production of œstrin in sub-minimal amounts with the periodic attainment of a threshold value.

The arguments for and against these three possibilities are discussed below.

(1) The conception that the sterilised ovaries elaborated œstrin periodically is somewhat similar to the theories hitherto held with regard to the regulation of the œstrous cycle in normal animals. It has usually been assumed that œstrin is produced cyclically in normal ovaries by means of its elaboration being confined to the maturing Graafian follicle. In the sterilised ovaries the problem is complicated by the apparent absence histologically of any cycle, and it is doubtful whether the cyclic production of the hormone could be brought about without this. Any views, moreover, based on the idea of cyclic production, whether in normal or sterilised ovaries, are very difficult to reconcile with (*a*) the fact that œstrin can be obtained practically from any ovary, even ovaries from pregnant or anœstrous animals, and (*b*) the experimental observation previously reported in conjunction with Mr. Bellerby (7), that œstrin can be obtained from the non-cyclic stromal tissue of normal ovaries.

(2) The idea that in the sterilised ovaries the œstrous-producing stimulus is constantly present, but is periodically inhibited by some œstrous-inhibiting factor, is analogous to Hammond's (3) view of the œstrous cycle in normal animals as regulated by the corpus luteum. It seems impossible, however, to apply such a view to the regulation of the œstrous cycle in the sterilised animals, and, moreover, the occurrence of the cycle in these sterilised animals is a serious obstacle to the acceptance of Hammond's views relating to the part played by the normal corpus luteum in the regulation of the œstrous cycle in the normal animal. The absence of periodic œstrous-inhibitor effect in the sterilised ovary may be concluded from two facts :—

(i) It is difficult to suppose that the histologically constant tissue of the sterilised ovary can elaborate œstrin and, in addition, periodically produce an inhibitor substance.

(ii) In a minority of cases only is any tissue comparable with normal corpus luteum tissue present, and this is fundamentally different in not being cyclic. This tissue may be derived from atretic follicles and be in the form of persistent corpora lutea atretica, or it may constitute practically the whole ovary as transformed tissue of the first post-irradiation ingrowth (Group 3).

In the former case no influence on the cycle is detectable, while in the second case the cycle seems to be inhibited permanently by the mechanical crowding out of the tissue responsible for the elaboration of the œstrous-producing stimulus.

(3) While it is not impossible that future detection of a histological cycle in the sterilised ovaries may necessitate modification of these present views, it seems necessary at the moment to suppose that the cycle in the sterilised mouse is brought about by the periodic attainment of a threshold value on the part of the constantly elaborated œstrous-producing stimulus. The serious objection to this is, of course, the necessity for assuming that after œstrus has been produced, the concentration sinks below the threshold value, and this implies that relatively large amounts of œstrin are used up in the production of œstrus. Nevertheless, some such theory seems inevitable in the present state of the data relating to this sterilised material.

The Regulation of the Œstrous Cycle in the Normal Animal.—Finally, it is necessary to consider what light, if any, is thrown by these experiments on the regulation of the œstrous cycle in the normal intact mouse. In the first place it is possible to say quite definitely that in view of these results it is necessary to reconsider the accepted view that the alternating presence of mature follicles and corpora lutea in the normal ovary is essential for the production of the œstrous cycle. It seems quite evident that they are not essential, and on this view the intimate association of œstrus and follicular maturation is accounted for, not because the latter is the exciting event, but because both are expressions of an ovarian rhythm. From this standpoint the corpus luteum of ovulation in the unmated mouse is a purely tentative organ, functionless as regards œstrus-inhibition if pregnancy does not ensue, but capable of taking on an inhibiting function if caused to become persistent by pregnancy. In certain cases, however, the corpus luteum of ovulation may apparently become semi-persistent and exert an œstrus-inhibiting action, as after sterile copulation in the mouse and rat. This may be analogous to the results of Hammond (3), who found that œstrus in the cow could be expedited by squeezing out the corpora lutea of the previous ovulation before it was due. The relation of the X-ray work to the experiments on the extraction of "œstrin" from ovaries has been discussed before (7) and need not be entered into here.

VII. *Summary.*

(1) Further evidence is brought forward in support of the conclusion previously arrived at, *i.e.*, that all the normal cyclic œstrous phenomena except ovulation can occur in animals in which the Graafian follicles of the ovary have been totally destroyed at an early age by exposure to X-rays, and can occur, therefore, in the absence of organised corpora lutea and Graafian follicles.

(2) The main series of animals described in this present paper were irradiated immediately after birth. A smaller series, of which only one sterilised animal survived, were irradiated as fœtuses *in utero*. In the two series, 63 periods of œstrus were observed in 24 sterilised females.

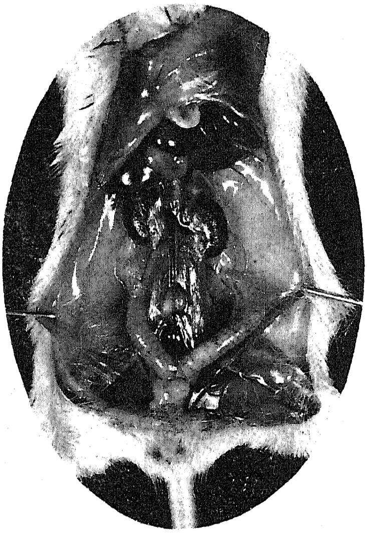
(3) Nine of these animals, however, showed only six periods in all, and these animals were found to have ovaries composed almost entirely of luteal-like tissue, and since the absence of the œstrous cycle cannot be directly ascribed to the absence of follicles, it is supposed that its absence in these animals was due to the direct mechanical inactivation of the normal sterilised ovary by the destructive development of this luteal-like tissue.

(4) The observation of 57 periods of œstrus in the other 15 sterilised females, whose ovaries possessed neither Graafian follicles nor corpora lutea vera, is direct supplementary evidence that neither of these structures is essential for the regulation of the cyclic phenomena which constitute the œstrous cycle of the accessory reproductive organs, and, taken into conjunction with previous results, seems to prove this point conclusively.

(5) The light thrown by these experiments on the regulation of the normal œstrous cycle is discussed.

Addendum.

After this paper was written I received from Dr. Schubert, of Berlin, the typescript of a paper describing similar experiments with similar results on the adult animal. Dr. Schubert's work will be more adequately mentioned in Part III of the present series, but I wish to take this opportunity of expressing my appreciation of his courtesy in allowing me to read his paper before publication.—*December*, 1926.



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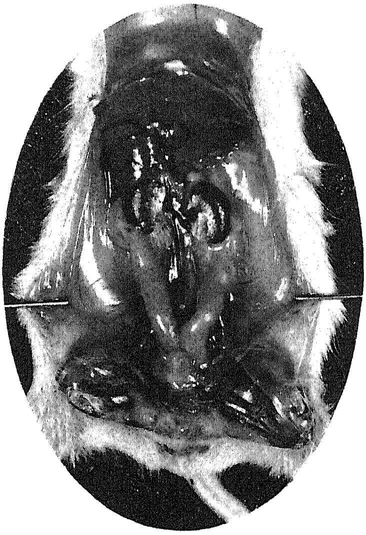

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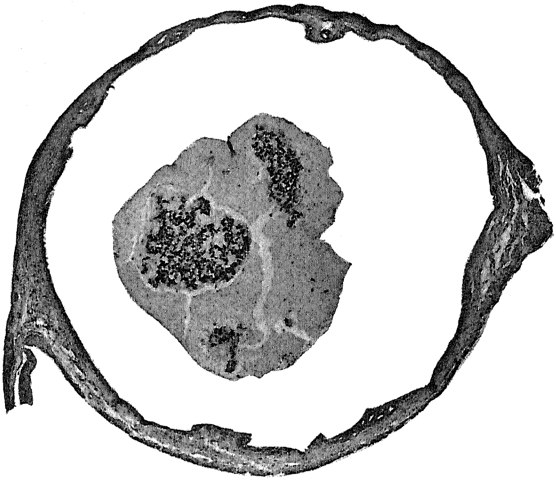

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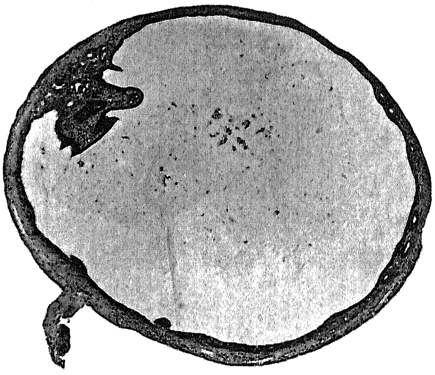

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DESCRIPTION OF PLATE 6.

FIG. 1.—Dissection of BX 16, showing typical œstrous condition of uterus.
FIG. 2.—Dissection of BX 75, showing typical œstrous condition of uterus.
FIG. 3.—Uterus of BX 16, showing œstrous inflation.
FIG. 4.—Uterus of BX 75, showing œstrous inflation.

I am indebted to Mr. F. J. Pittock for the photographs reproduced in figs. 3 and 4.

Changes in the Ovary of the Mouse following Exposure to X-Rays. Part II.—*Irradiation at or before Birth.*

By F. W. ROGERS BRAMBELL, Fellow of the International Education Board, A. S. PARKES, Beit Memorial Research Fellow, and UNA FIELDING.

(Communicated by Prof. J. P. Hill, F.R.S.—Received October 22, 1926.)

[PLATES 7, 8 and 9.]

(From the Department of Embryology and Histology, the Department of Physiology and Biochemistry and the Department of Anatomy, University College, London.)

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I.—INTRODUCTION.

The ovaries of a series of mice X-rayed at weaning-time (3 weeks old) were described in a previous paper (2), and the œstrous cycles of these animals were dealt with elsewhere (Parkes, 4). Although all the ovaries of the animals described in the latter paper were devoid of follicles and the majority of corpora lutea, normal œstrous cycles were observed in almost all cases. Histological study led us to conclude that following irradiation :—

(1) All the oocytes degenerated completely, and the cells of the membrana granulosa, and of the theca interna, in follicles where it was differentiated, followed their example shortly afterwards. In a few cases the larger follicles did not degenerate after the disappearance of the oocyte but formed corpora lutea atretica which persisted but had no effect on the cycle.

(2) The germinal epithelium proliferated cords of cells, which pressed the remnants of the old stroma into the medullary region. These cords formed the major part of the irradiated gonad and were probably responsible for the production of the œstrus-producing hormone "œstrin."

(3) The germinal epithelium in some cases proliferated a second lot of cords, which resembled structures described by some authors as anovular follicles. They resembled more closely, however, the "tubules" of some free-martin testes and structures described in the rabbit ovary as "spermatic tubules" (Hammond, 3). These cords were shown to have no influence on the œstrous cycle.

The present series of experiments were carried out on animals of a different age, with a view to corroborating, if possible, these results from a different standpoint. It was hoped that the follicles might be removed more completely and satisfactorily, and that the formation of corpora lutea atretica might be avoided by irradiation at or before birth, as follicles are scarcely formed in the ovary of the mouse at this time. This expectation was unfortunately not realised. Further, it was thought that the more actively growing gonad of the fœtal or new-born mouse might exhibit more extreme changes than those of the 3 weeks old mouse. Finally, since the accessory organs of reproduction are so little differentiated until some time after birth it was to be expected that the effect of the irradiated gonad on them would be more easily demonstrated.

The full details of the œstrous histories of these animals are given in a paper by one of us (5) and the accessory organs are described in another paper (6). The previous literature is summarised in these papers and in that on the ovaries of the mice X-rayed at 3 weeks old (2).

II.—HISTOLOGICAL DESCRIPTION OF CASES.

The material was fixed in Bouin's or Carnoy's fluid and stained with Ehrlich's hæmatoxylin, or in Champy's fluid and stained with Heidenhain's iron-hæmatoxylin.

(*a*) *Animals X-rayed before birth.*

The animals described in this section were irradiated *in utero*, the mothers being exposed to the X-rays during the later stages of pregnancy. The mothers, belonging to the series of PX's and SX's, do not come within the scope of the present paper, but will be dealt with in a subsequent one on the ovaries of mice irradiated when adult. Much of the original testing and standardisation of doses was carried out on these animals. The consequent irregularity of results is accentuated by the differential screening of the fœtuses by one another, and by the bony and vascular tissues of the mothers and of the placentæ. For these and other reasons there are only six animals worth describing here. Of these FPX 10*a*, *b*, 6*a* and 8*a* were given one 40 min. dose, the first three one day and the fourth four days before birth. FPX 10*a*, *b*, and 8*a* were killed 6, 13 and 21 days after birth respectively. FPX 6*a* was mated when two months old and killed when three months old. It was observed to have three œstrous periods. FSX 2 was given two 10 min. doses, two and five days before birth respectively. It was killed when adult. FSX 3 was given three 10 min. doses 2, 5 and 9 days before birth respectively. It was killed when adult and proved to have very small ovaries, although the capsules and uteri were well developed. FPX 10*a* and *b* have not as many oocytes as is normal, and those present are all surrounded by a single or double layer of follicle cells. The follicles are all in the periphery, the medullary region being occupied by somewhat hypertrophied stromal tissue. The ovaries of FPX 10*b* are larger than those of 10*a*, but otherwise not very different. Neither exhibit any definite rapid proliferation of new cords from the germinal epithelium. FPX 8*a* is similar to the normal three-weeks old ovary, except that it contains no small oocytes, and some full-sized ones are present in the stroma with practically no follicles around them. Many of the oocytes exhibit mitotic spindles, cleavage and fragmentation, and other signs of degeneration.

The ovaries of FPX 6*a* (Plate 8, fig. 4) are very similar to those of many of the animals X-rayed at birth. They contain some small cavities, enclosing shrunken zonæ pellucidæ, which represent former follicles and are similar to those described and figured in a previous paper (2). Some of the epithelial canals, to be described later, are also present. The remainder of the tissue is rather luteal-like, but the fixation does not permit of it being decided whether this is hypertrophied stromal tissue or cords of the first post-irradiation proliferation, nor is it possible to be sure whether corpora lutea atretica are present or absent. There are, however, no oocytes or follicles present. FSX 3 is similar, but definitely does not contain corpora lutea atretica. One ovary contains an abnormally large follicle, with a mature oocyte in the discus proligerus and an enormous liquor-filled antrum, and another small follicle with an oocyte. The ovaries of FSX 2 contain many apparently normal follicles but also many obviously degenerate ones. Both ovaries contain a large number of corpora lutea atretica, which, with the follicles and a small amount of connective tissue, constitute their entire substance (Plate 7, fig. 2). In a single section of one ovary as many as 25 can be counted. The ovary, increased in bulk by their presence, measures 2·7 mm. and 2 mm. thick.

Only one of these 6 animals, FPX 6*a*, was completely sterilised and entirely lacked follicles, yet it was observed to have three œstrous periods during adult life. These animals also show that although the majority of the oocytes disappear within one week after irradiation, certain follicles grow slowly and produce corpora lutea atretica which persist in the ovary. It must be remembered that in the full-time female mouse fœtus the germ cells are scattered among the future follicle cells in the sex-cords, and that the latter do not arrange themselves around the oocytes until about birth. Those oocytes which have escaped immediate destruction by the X-rays have therefore to grow and acquire follicles, which finally produce corpora lutea atretica. This growth of the oocyte and follicle, though abnormally rapid, extends over a considerable period. The irradiated ovary six days after birth (FPX 10*a*) is about the size normal for this age, and the oocytes and follicles in it are all about the size and stage of development of the larger ones in the normal week-old mouse ovary. The same is approximately true of the irradiated ovary 13 days after birth (FPX 10*b*), except that it is markedly below the normal size. At three weeks after birth the irradiated ovary is smaller than the normal, but all the follicles in it are at the same stage as is normal, except that some full-grown oocytes are practically devoid of any follicle at all. At all three ages the irradiated ovary is remarkable in that it does not contain any small oocytes, which are found in large numbers in the cortex of the normal ovary between birth and puberty. In consequence the total number of oocytes in the irradiated ovary is small.

It is obvious, although intermediate stages are lacking in this series, that some of the follicles still present in the irradiated ovary three weeks after birth produced the corpora lutea atretica found in the adult ovary of FSX 2 (Plate 7, fig. 2). This proves that although irradiation before birth destroys the majority of the oocytes, others grow and form follicles, which persist as such for more than three weeks and some of which subsequently form corpora lutea atretica which are found in some ovaries when adult, and which still exhibit the effects of irradiation in their persistent nature.

It is unfortunate that these animals throw no light on the question of the origin of the tissues of completely sterilised ovaries, such as those of FPX 6*a*, and one ovary of FSX 3. None of the series exhibit any active proliferation of epithelial cords, and, if these are formed, it presumably takes place more than 3 weeks after irradiation.

(*b*) *Animals X-rayed at birth.*

The thirty animals described in this section were given a 40 min. irradiation during the first three days after birth, and a second similar dose one week after. The results were remarkably uniform, only one animal being found to be incompletely sterilised. For the purposes of calculation of the time after irradiation when they were killed, the date of the first dose is used in all cases. Six of the animals (BX 42, 44, 37, 35, 34 and 33) were killed at periods of 10, 14, 21, 28, 35 and 42 days after irradiation respectively, and form a series which will be described first. The other 24 animals became mature and the vaginal contents were observed daily for varying periods before being killed. The vaginal smear technique employed, together with the œstrous histories of these animals are described elsewhere (5) and need not be repeated here. It is convenient to class the ovaries of these animals under three groups, according to their histological characteristics, for purposes of description.

Most of the material was fixed in Bouin's fluid and stained with Heidenhain's iron-hæmatoxylin or with Ehrlich's hæmatoxylin and eosin. Some of the ovaries of the series were fixed in Champy's fluid and stained with Heidenhain's iron-hæmatoxylin. Both methods gave very satisfactory results. Complete serial sections were made in all cases.

The Series.—The first animal of the series, BX 42, was killed 10 days after irradiation. The ovary contained some apparently normal follicles about the size of the larger ones found in the normal ovary at this stage (Plate 8, fig. 1). It differed from the normal, like the animals considered previously, and all those to be subsequently described, in containing no traces of small oocytes. Epithelial cords were present in the periphery, outside the follicles, and were being formed by proliferation from the germinal epithelium, connective tissue cells of the ovary contributing a thin sheath to them as they pushed in.

Their rapid formation and growth had disturbed the albuginal elements to such an extent that they did not constitute a definite tunica, except near the hilus. These new cords appear to be outside, separate and distinct from the "cords" in which the follicles were situated. The next specimen, BX 44, was killed 14 days after irradiation. There are few oocytes in the ovary, and they have larger follicles, with two to five layers of membrana granulosa elements, but no differentiated theca interna. The largest follicle has a small antrum. Many of the follicles show signs of degeneration, as do the majority of the oocytes. It is difficult to be sure whether the small cords and groups of epithelial cells found in the periphery between the follicles have originated from the epithelium before or after irradiation, as the germinal epithelium is not very active, though proliferating in places, and the tunica albuginea is fairly well formed. BX 37, killed 21 days after irradiation, has a number of follicles in all of which the membrana granulosa is several cells, and in some is abnormally, thick. The larger follicles have a large antrum and a distinct theca interna. All the oocytes and some of the follicles seem to be degenerating. The small amount of stromal tissue between the follicles appears unhealthy and vacuolated, and there are no newly-proliferated cords, except in one or two places where the germinal epithelium is active. Otherwise the germinal epithelium is thin, flattened and inactive, and the tunica albuginea is thin and continuous.

BX 35 was killed 28 days after irradiation. A large part of the ovary is occupied by an enormous follicle, 0·35 mm. in diameter. In it the membrana granulosa is abnormally thick on the side of the discus proligerus and the antrum is abnormally large. The theca is unduly thick and contains numerous glandular theca interna elements. The germinal epithelium is thickened and actively proliferating cords, similar to those in BX 44, which form a peripheral zone around the single follicle. These cords derive their connective tissue sheaths from the elements of the tunica albuginea, which is much disturbed by their ingrowth through it. BX 34 was killed 35 days after irradiation. One ovary contains one and the other two large follicles 0·35 to 0·46 mm. in diam. The oocytes in them are degenerating and the membrana granulosa thickened. The theca interna is well developed and two of the follicles are filled with blood. Around the follicles are some loose vacuolated cells, which seem to be the remnants of the old stroma, and outside these a peripheral zone of new epithelial cords similar to those in BX 42 and 35.

The last of the series, BX 33, was killed 42 days after irradiation. One ovary contains four and the other one retrogressing corpus luteum atreticum. Neither ovary contains any follicles or oocytes. Outside the corpora lutea atretica are new cords of epithelial cells, which are still being proliferated from the germinal epithelium in places. These cords are larger and better developed than in BX 42, 35 or 33, and the epithelial cells are slightly larger and more glandular looking. They are more developed in the ovary that has only one corpus luteum atreticum, and in it the epithelial cells are somewhat luteal-like in places, with a large smooth amphophil cytoplasm. The tunica albuginea is well developed but is disturbed in places where cords are still being proliferated.

Adult Animals. Group I.—The animals of this group, seven in all, are characterised by the vacuolated cytoplasm and puckered nuclei of the epithelial cells of the first proliferation. These cells appear unhealthy and shrunken and differ in this respect from the large, vacuolated, round nucleated cells of BX 70, 69 and 31. Fibroblasts are rather more numerous also in these ovaries than in those of either of the other groups, and suggest a tendency towards fibrosis, which has, however, nowhere proceeded far.

BX 77 was killed 14 days after the only œstrous period observed. The ovaries contain no oocytes or follicles, but one contains one and the other three small retrogressing corpora lutea atretica. The chief part of both ovaries consists of the tissue of the first proliferation in the condition typical of this group (Plate 7, fig. 6, and Plate 9, fig. 3). The cells are rather smaller and more vacuolated than those of the other specimens. The germinal epithelium is thickened and in some places almost columnar and the tunica albuginea is very well developed. There are a few tubules of the second proliferation, similar to those described in the M series, in the periphery. BX 32 was killed 24 days after the second œstrous period observed. The vaginal content was cornified for the last 20 days. One ovary contains a single retrogressing corpus luteum atreticum. Both ovaries are chiefly composed of the typical vacuolated tissue of the first proliferation, which, except for one area in the ovary without the corpus luteum atreticum, is not so small and shrunken celled as is BX 77. BX 62 was killed 13 days after the only œstrous period observed, the vaginal contents being cornified continuously for that time. No oocytes, follicles or corpora lutea atretica are present. The cells of the first proliferation are arranged in cords more definitely than in the two previous cases. They resemble them, however, but are not so vacuolated, and in places slightly resemble the luteal-like cells of the next groups. Fibroblasts are plentiful. The germinal epithelium is cubical or slightly flattened and is forming some intermediate type cords of the second proliferation, a few of which are scattered around the periphery.

BX 88 was killed two days after the second œstrous period observed, but the vaginal content was a good deal cornified all the time it was being examined. One ovary contained several corpora lutea (vera or atretica) in various stages of retrogression and a single small follicle. The other contained one corpus luteum atreticum. This animal is the only one of the 24 adults described in which an oocyte or follicle was present. Both ovaries had large cysts and many epithelial canals similar to those described in M 16. The tissue of the first proliferation was very similar to that in BX 32, the cells in some regions being more shrunken than those in others. BX 16 was killed during the ninth œstrous period observed. Both ovaries contain one very large and some small cysts and many epithelial canals. One ovary has a healthy corpus luteum atreticum, but that present in the other is becoming fibrosed with picnotic cells among the fibroblasts. The cells of the first proliferation are similar to those in the previous cases but tend to be somewhat luteal-like and rather larger. BX 91 was killed not more than 18 days after the only œstrous period observed. One ovary contains a large cyst and both are riddled with epithelial canals. The tissue of the first proliferation is composed of vacuolated cells similar to the larger one in BX 32. The germinal epithelium is thick and convoluted, but does not appear to be actively proliferating cords, and the tunica albuginea is excessively developed. BX 90 was killed 18 days after the only œstrous period observed. The ovaries are similar to those of BX 91, except that the germinal epithelium and tunica albuginea are normal.

Group II.—The eight animals comprising this group are characterised by the cells of the cords of the first proliferation which are slightly enlarged and glandular looking. The cytoplasm is smooth and little vacuolated, and the nuclei are round and healthy and often exhibit mitotic figures. Fibroblasts are not plentiful, and the tissue as a whole appears young and healthy. It has a distinct resemblance to luteal tissue, but the similarity is not nearly as great as in the next group. All the animals are completely sterilised, none containing any oocytes or follicles.

BX 64 was killed one day after the fourth œstrous period observed. The ovaries contain no corpora lutea atretica but some small cysts and many epithelial canals are present. The cords of the first proliferation constitute the major part of the ovaries; their cells have slightly enlarged after proliferation from the germinal epithelium and show a tendency to become luteal-like. The tunica albuginea is fairly well developed and the germinal epithelium is not actively proliferating, though a few tubules of the second proliferation are present in the periphery. BX 46 was killed four days after the 13th œstrous period observed. No corpora lutea atretica are present, but a few small cavities (similar to those figured in 2) filled with a pink-staining coagulum probably represent the remains of follicles. The cells of the first proliferation are similar to those described in BX 64. The germinal epithelium is thickened and actively forming very many beautifully developed tubules of the second proliferation which form a broad peripheral zone, especially well seen in one ovary. The tunica albuginea is disturbed by the new tubules. BX 63 was killed two days after the 2nd œstrous period observed. One small cyst is present in one ovary, both ovaries being otherwise similar to those of BX 46.

BX 75 was killed during the 3rd œstrous period observed. The ovaries contain no cysts and the tunica albuginea is well developed (Plate 7, fig. 4). The cells of the first proliferation are rather larger and more luteal-like than in BX 63, which the ovaries otherwise closely resemble (Plate 8, fig. 3). BX 15 was killed 6 days after the 2nd œstrous period observed. Some small cysts and a few epithelial canals, but no corpora lutea atretica are present in the ovary. The cells of the first proliferation resemble those of BX 75. The germinal epithelium is proliferating but not very actively, and the tunica albuginea is poorly developed. Some well-developed cords of the second proliferation are present in the periphery, but they are not so plentiful as in the three previous cases. BX 56 was killed 9 days after the 5th œstrous period observed. Two or three old corpora lutea are present in the centre of the ovary, and one fairly large cyst. It is otherwise similar to BX 15, except that the tunica albuginea is thick and well developed. BX 76 was killed 3 days after the 5th œstrous period observed. Both ovaries contain 3 or 4 old corpora lutea atretica. The cells of the first proliferation are similar to those in BX 75. The germinal epithelium is thick and columnar in places, and the tunica albuginea is thick but not very fibrous. There are some cords of the second proliferation in the periphery. BX 89 was killed 3 days after the 4th œstrous period observed. One ovary contains large cysts and both many epithelial canals. There are no cords of the second proliferation and the cells of the first proliferation are similar to those of BX 75.

Group III.—The nine animals comprising this group are characterised by the large very luteal-like cells of the first proliferation. They seem to arise as a further stage in the luteal-like development of this tissue, initiated in the previous group. In most the cells are not much vacuolated and the cytoplasm appears smooth, although on close examination it is found to be finely granular, a characteristic of true luteal-cells. In the other cases the cells are much vacuolated, probably by fatty infiltration, but by their size and general characters are still easily identified as belonging to this group. Fibroblasts are not plentiful and the tissues appear healthy, not shrunken as in the first group. All the animals are completely sterilised, none containing any oocytes or follicles.

BX 24 was killed 38 days after the second œstrous period observed. The ovaries contain no cysts or corpora lutea atretica, but many epithelial canals are present. The cells of the first proliferation have deeply basophil round nuclei and a large cell-body. The cytoplasm appears smooth, though really finely granular, and stains amphophil with an acidophil preponderance. Some of the cells are slightly vacuolated, but all appear healthy and are strikingly like luteal cells, far more so than those in any of the cases previously described. The germinal epithelium is thin and inactive on the whole, although a few cords of the second proliferation, rather intermediate in type, are present in the periphery. The tunica albuginea is very poorly developed and is practically absent in places. BX 51 was observed daily for 37 days before being killed, but no œstrous periods occurred. The ovaries are similar to BX 24, except that in places the cells are even more luteal-like and some giant-cells occur (Plate 8, fig. 2). The giant-cells in extreme cases measure as much as 40 μ in diameter. Their cytoplasm is similar to that of the other luteal-like cells, of which they appear to be abnormal hypertrophied individuals. The nuclei do not enlarge in proportion to the cytoplasm and never measure more than 12 μ in diameter. They usually appear crenated and unhealthy and one was observed to have a double nuclear membrane. Some of the luteal-like cells appear to be bi-nucleate. BX 25 was observed daily for 36 days before being killed, but no œstrous periods occurred. Many epithelial canals are present. The cells of the first proliferation are large and very luteal-like and resemble those of the last two cases. The germinal epithelium appeared inactive and the tunica albuginea thin and poorly developed.

BX 50 was killed 39 days after the only œstrous period observed. The ovaries of this animal are larger than those of any other adult BX, one measuring on the slide 1·86 × 1·20 × 1·14 mm. They contain no cysts but many epithelial canals. The germinal epithelium is inactive and the tunica albuginea poorly developed, except where these canals are forming. Some epithelial cells and many fibroblasts occur between these canals and appear to have come from the germinal epithelium and tunica albuginea. The tissue of the first proliferation is very luteal-like and is similar to that in BX 25. BX 13 was observed daily for 36 days before being killed, but no œstrous periods occurred. The ovaries contain many epithelial canals (Plate 7, fig. 3). The tissue of the first proliferation is extremely luteal-like (Plate 9, fig. 1); more so than in any other case except BX 14. The germinal epithelium is thin and inactive and the tunica albuginea is scarcely developed at all. BX 14 has an identical history and similar ovaries to BX 13. BX 70 was killed 48 days after the second œstrous period observed. The ovaries contain several small retrogressing corpora lutea atretica and many epithelial canals. The tissue of the first proliferation is large celled and very luteal-like, but the cells are more vacuolated than in the cases already described in this group, and were probably infiltrated with fat. An area of this tissue in each ovary is being invaded by fibroblasts and is not unlike that described in the first group, except that the luteal-like cells are larger and appear healthy. Apart from the formation of epithelial canals the germinal epithelium and tunica albuginea are thin and inactive.

BX 69 was killed 65 days after the only œstrous period observed. The ovaries contain no corpora lutea atretica, but many epithelial canals are present. The tissue of the first proliferation (Plate 7, fig. 1, and Plate 9, fig. 2) is similar to BX 70, but fibrosis, though indicated in places, is nowhere extensive. BX 31 was observed daily for 36 days before being killed, but no œstrous periods occurred. The ovaries were rudimentary, being smaller than those of any other adult BX, and measuring on the slide 0·6 × 0·5 × 0·75 and 1·0 × 0·37 × 0·51 mm. respectively. One ovary contained a small cyst. The small amount of tissue present appeared to consist of cells of the first proliferation, which were large, luteal-like and vacuolated, especially in one ovary, where they resembled those of the two previous cases, BX 70 and 69.

III.—General Histological Description.

(i) *Histological Changes following Irradiation. The follicles.*—In the ovary of the mouse at birth the oocytes are arranged in the cords of the cortex in four or five layers. The largest of these, those in the layer nearest the centre, are surrounded by three or four flattened epithelial elements derived from the cords. These form the first rudiment of the follicle. Before the end of the first week almost all the oocytes have follicle cells around them, and in the case of the deeper ones these form a continuous single or double layer of cubical cells, while the fibroblastic elements of the cords have formed a thin thecal coat around them. Irradiation, in suitable amount, shortly after birth results in the immediate and total destruction of the majority of the oocytes, no traces of which can be seen after 10 days (Plate 8, fig. 1). This is similar to the effects on those X-rayed *in utero* and at 3 weeks old. Again, as in these cases, a certain number of the oocytes seem, however, always to escape this sudden destruction and to grow in an apparently normal manner for a considerable time. At ten days after irradiation all the oocytes present have approximately completed the growth stage and are surrounded by well formed follicles. They are in fact at the stage of the large follicles found at this age in the normal mouse. At 14 days after irradiation the oocytes and their follicles have grown to the size of the larger follicles normally found in the fortnight old mouse; and at 21 days the follicles have reached the largest size normal for that age, but all the oocytes seem to show signs of degeneration, and in some of the follicles the membrana granulosa is abnormally thickened. From this time on the changes are degenerative and may follow several courses. First, after the oocyte has disappeared, all but the zona pellucida, the surrounding membrana granulosa cells and the elements of the theca externa, when it is differentiated, may break down. Nothing then persists but the shrunken zona pellucida in a cavity surrounded by a thin fibrous wall. This process is similar to that commonly observed in the normal mouse ovary, and has been described and figured by us in the previous paper (2). In the present series of animals these cavities have only been observed in a few of the adult animals, and in the majority they have disappeared completely. Secondly, after the degeneration of the oocyte a follicle of any size may become cystic. The resulting cysts, found in many of the animals, vary in size from that of a small follicle up to the size of the whole of the rest of the ovary. They are filled with fluid and lined by a thin cubical epithelium, outside of which is the thin fibrous wall. Sometimes follicles about to become cystic grow to an enormous size, as much as twice the maximum normal diameter. Blood capillaries in the wall then burst and fill the antrum with extravasated blood. Then oocyte and membrana granulosa break down and the cyst is formed as has been described previously (2). Thirdly, the follicles may become atretic, the oocyte degenerating and the cells of the membrana granulosa and theca interna invading the cavity and forming a corpus luteum atreticum. Whichever the fate of the follicles they have all disappeared as such by the 6th or 7th week after irradiation.

Corpora lutea atretica, formed from atretic follicles, are found in a number of animals, BX 77, 32, 88, 16, 56, 76, 70 and 33. They are in all stages of degeneration and transformation into corpora fibrosa, but some are apparently healthy. In some cases a very retrogressive one may be found beside one showing no signs of retrogression. They appear to be very persistent and in some cases may be considered a permanent part of the irradiated ovary.

The Stroma.—The changes which take place in the stroma seem to be identical with those described in the M series X-rayed at weaning time. In the BX animals, however, they are by no means so clear; thus the more or less complete degeneration of the old stroma and its replacement by the new tissue proliferated by the germinal epithelium are less emphatic. It appears, however, that the old stromal tissue does not increase much in volume after irradiation, and that the growth of the ovary after the degeneration of the follicles is due almost entirely to the tissue proliferated from the epithelium. The first proliferation of the germinal epithelium begins soon after irradiation and appears to continue for a considerable time, being apparent after 10 days (Plate 8, fig. 1) and not having been completed after 42 days. The cords are fewer and rather smaller, at first, than in the M series but are otherwise similar. No separate description is necessary here as that in the previous paper (2) is applicable to them also. They seem to have completed their formation before the animal becomes adult. Unlike the M series the BX mice can be easily assigned to three groups by the characters of the tissue of the first proliferation. The shrunken vacuolated cells that form this tissue in the first group (Plate 9, fig. 3) probably arose as a comparatively early alteration in the then not much hypertrophied epithelial elements of the cords, the tendency to fibrosis being another sign of retrogressive changes. The cells of the second group suggest that in these aninals the tissue instead of becoming vacuolated, continued to grow actively and to become luteal-like, with dense cytoplasm and rounded nuclei (Plate 8, fig. 3). In the third group this tendency became more marked and the tissue became so luteal-like that cell for cell it could hardly be distinguished from true luteal tissue (Plate 9, fig. 1), although the arrangement in thick finger-shaped cords, not large spherical bodies, furnished a ready means of distinguishing it. This luteal-like character of the tissue reaches its acme in BX 13 and 14. In BX 70 and 69 the tissue (Plate 9, fig. 2), obviously very luteal-like before, and still retaining its characteristics, is becoming vacuolated, probably by infiltration of fat. This condition appears to be the extreme condition of the tendencies exhibited by the cases in Groups II and III.

The germinal epithelium, as in the M mice, appears to become thin and inactive after the proliferation of the first cords has ceased. The tunica albuginea, disturbed by their ingrowth, again becomes continuous and thin.

Cords of the second proliferation are not nearly as plentiful as in the females X-rayed at weaning-time, and only occur in less than half the cases, notably BX 77, 62, 64, 46, 63, 75, 15, 56, 76, 24 and 51. They are not numerous in any of these, except BX 46, 63 and 75 (Plate 8, fig. 3) and in none are they nearly as plentiful or well formed as in M 6*e*. Their structure and mode of origin is similar to those in the M series, and need not be described again here.

(ii) *Topographical Changes following Irradiation.*—The initial sudden destruction of oocytes does not result in a marked decrease in size of the ovary as a whole, like that occurring in the cases X-rayed at 3 weeks old. This is obviously due to the small size of the oocytes and the practically total absence of follicles in the new-born mouse. The subsequent growth appears to be fairly steady, but the actual size of the ovaries in the adult varies very much, though all are considerably smaller than those of the normal adult. The maximum size of the ovary on the slide is exhibited by BX 50, one ovary of which measured 1·86 × 1·20 × 1·14 mm. The minimum size of 0·6 × 0·5 × 0·75 and 1·0 × 0·37 × 0·51 mm. by BX 31. There is no noticeable size difference between the three adult groups.

The hyperæmic congestion remarked in the M series is also common, indeed the rule, among the BX series. The arrangement of tissues is essentially the same as in the M mice also. The loose vascular medullary zone in the hilar region probably contains what little of the pre-irradiation stromal tissue remains. Outside this is the main mass of the ovary composed of cords of the first proliferation. A peripheral zone of cords of the second proliferation is only present in some animals however. This general arrangement is somewhat disturbed by the occurrence of epithelial canals in more than half of the cases, of cysts in about a third and of corpora lutea atretica in about the same number.

(iii) *Anomalous Structures found in Irradiated Ovaries.*—The method of origin and structure of the cysts have already been described in this paper and elsewhere (2). It is worth noticing that more than half the animals in Groups I and II have cysts, whereas only one small one is present, in BX 31, in Group III. The epithelial canals which riddle the ovarian tissue of so many animals and are figured in Plate 9, fig. 1, are similar to those described in M 15, 16 and 42, but are much longer and more numerous. We can offer no explanation of their cause, but they are produced by invaginations of the germinal epithelium carrying down with them a thin connective tissue sheath from the tunica albuginea. The cubical epithelium forming their lining is continuous with the germinal epithelium. They are circular in cross section and their lumen is open and communicates with the cavity of the capsule. They ramify into the deepest portions of the ovary, but do not penetrate the tissues of corpora lutea atretica although they may grow close around them. This suggests that they are formed at a late stage, after the corpora lutea atretica.

IV.—DISCUSSION.

(*a*) *The Regulation of the Œstrous Cycle.*

It was shown that female mice X-rayed at weaning-time (4) developed an œstrous cycle when adult in the normal manner although the ovaries were devoid of follicles and corpora lutea vera. Since double ovariotomy invariably stopped the œstrous cycle, the ovaries of these mice were clearly responsible for the production of this œstrous cycle. The tissue composing the ovary arose by two proliferations of epithelial cords from the germinal epithelium after irradiation. It was shown that the tissue derived from the first post-irradiation proliferation was probably responsible for the production of the œstrous cycle, and that the cords of the second proliferation did not affect it at all.

The present series of animals, X-rayed at a different age, entirely support these conclusions. Twenty-three of the twenty-four adult BX's described were devoid of follicles, and only six of these had corpora lutea atretica, all of which were persistent. Eighteen of these 23 animals had at least one œstrous period and 59 periods were observed altogether.

The reasons for assuming that the tissue of the first proliferation is responsible for the production of œstrin are the same as in the previous series. Briefly, in the absence of follicles, corpora lutea and almost all traces of the old stromal tissue, the new components may be assumed to have taken on the rôle of œstrin production. Among these components only the tissue of the first proliferation is sufficiently regular in its development to account for the distribution of the phenomena. It is obvious that the cords of the second proliferation, corpora lutea atretica, cysts and epithelial canals are not responsible as they are not present in many animals with cycles. Indeed, by the œstrous history alone the cases with these structures cannot be distinguished from those without, so we are entitled to assume that these structures have no effect upon the œstrous cycle. The evidence then is overwhelming that the tissue of the first proliferation is the chief, though possibly not the only, seat of œstrin production in the sterlilised ovary.

The present series of animals differs markedly from the M series in the number of animals which never exhibited an œstrous cycle, or did not do so for a long time previous to being killed. These phenomena are correlated in a remarkable manner with the histological condition of the cords of the first proliferation. In the first two groups all the cases exhibited at least one œstrous period, and in the longest case not more than 24 days previous to being killed.

Excluding the incompletely sterilised case, the 14 animals were observed to have a grand total of 53 œstrous periods; the average length of the last, previous to being killed, being only 7·4 days. In the third group 5 of the 9 cases never exhibited an œstrous period and the other 4 only exhibited 6 in all. None of these exhibited a period for at least 36 days previous to being killed.

It appears highly probable from these figures that the extremely luteal-like differentiation of the tissue of the first proliferation, characteristic of Group III, is not compatible with the maintenance of the œstrous cycle. It may be supposed that this is due either to the loss of the power of producing œstrin or to the production of an œstrin-inhibiting substance of the nature ascribed to the corpus luteum verum. The morphological similarity of this tissue suggests that it may have the same physiological properties. It is probable, however, that true luteal tissue in the mouse becomes functionally inactive if it persists for any reason for more than 3 weeks. There is no reason to suppose that this tissue would behave otherwise if it had similar physiological properties. Secondly, in BX 70, 69 and 31 the tissue is much vacuolated and probably infiltrated with fat, and might be expected to have ceased to function as luteal tissue, yet these animals had no cycle. In the latter case both ovaries were so rudimentary that they can hardly be supposed to have any important physiological effect. Thirdly, the infantile condition characteristic of the uteri of this group does not suggest that the ovaries are carrying on their normal endocrine functions with a simultaneous production and inhibition of œstrin. It would appear, therefore, that the cells of the cords lose the power of œstrin production when the differentiation into luteal-like cells has attained a certain point.

The eight cases composing the second group all had at least two œstrous periods, and the total for all was 38. The longest period between the last œstrous period and the time of killing was 9 days. This regularity in the œstrous history is what would be expected from the young, healthy and not over-differentiated condition of the cords.

The cases in Group I are more irregular. Apart from the incompletely sterilised animal, 15 periods were observed, but nine of these were in one animal, BX 16, which was killed during the last one. One animal was known to have the last period not more than 18 days before killing, but the exact time was not recorded. The remaining four animals had the last œstrous period 14, 24, 13 and 18 days respectively before killing. Two of the latter animals had the vaginal contents cornified for the last 20 and 13 days respectively, and may in that sense be said to have been on œstrus continuously for that time. Similarly the vaginal contents of BX 88, the incompletely sterilised case, were a good deal cornified all the time that they were under observation. It is possible that the degenerative changes observable in the cells of the cords of this group set in, for some reason, at an early stage, while they were still actively producing œstrin and before they had become much differentiated into the very luteal type of Group III. The data are, however, insufficient to draw any conclusions of importance.

(*b*) *The Morphological Significance of the Cords.*

The cords of the first and second proliferations are formed from the germinal epithelium, and their connective tissue sheaths are contributed by the elements of the tunica albuginea as they grow in. The second proliferation is distinct from the first and not a continuation of it, although the first formed cords are intermediate in character between those formed later and those of the first proliferation. The origin and nature of these cords have been discussed at length in the previous paper (2) and we do not propose to repeat them here.

The discussion of one or two points concerning the nature of the first cords has, however, been reserved for the present paper.

The similarity of differentiation of the cells of the cords of the first proliferation with those of an atretic or discharged follicle is remarkable, and the ultimate result, luteal-like tissue, is almost indistinguishable from true luteal tissue. This is of special interest because it is known that the follicle cells of the normal ovary are derived from the epithelial elements of the sex-cords. It appears therefore that in the irradiated ovary epithelial cells can produce luteal-like cells directly, while in the normal animal they have to pass through the follicular phase.

The present series of animals necessitates a consideration of the relations of the first post-irradiation proliferation with the organogenetic proliferations of the normal ovary. This problem also concerns the animals, previously described, which were irradiated at three weeks old. It would appear that the post-irradiation proliferations might correspond to the second or cortical and the third or definitive proliferations of the normal ovary, described in the cat by de Winiwarter and Sainmont (7). In fact the irradiation, while completely sterilising the ovary, might have stimulated the formation of the normal epithelial proliferations. This does appear probable, since similar proliferations do not occur in the mouse X-rayed when adult, as will be described in a subsequent paper of this series, where the question will be discussed further.

(c) *General Effects of X-Irradiation.*

Some general points concerning the effects of X-rays on the organism seem worth consideration here, although no explanation can be offered to account for them.

In some cases the growth of the animals X-rayed at birth was retarded and some of the cases appeared almost to be cretins. It was felt that this was probably due to disturbance of the endocrine system. Again, many of the animals became excessively fat when adult, the mesenteries, especially, being loaded with adipose tissue. It is interesting to note that of the 7 animals of the BX series exhibiting this, 6 (BX 50, 13, 14, 70, 69 and 31) were in Group III, and the other was BX 88, the incompletely sterilised animal. There appears, therefore, to be some correlation between the luteal-like tissue of the first proliferation and the fat metabolism, though it is difficult to see why these animals and not the others in Groups I and II should become so fat.

It is interesting that the effects of irradiation should, in some instances, become apparent almost immediately and in other cases only after long periods of time. Thus the oocytes either degenerate at once, as do the majority, or else they grow in an apparently normal manner for a considerable period and then degenerate weeks after irradiation. It is remarkable how full grown oocytes occur three weeks after irradiation in the cortex of FPX 8*a*, with practically no follicle around them. When the inter-follicular oocytes begin to degenerate, mitotic spindles and cleavages are often the first indications, as was noted in the animals killed at intervals after irradiation. It is still more remarkable that no new formation of oocytes from the cells of the germinal epithelium takes place after irradiation, although many have maintained (7) that this process continues after birth, 'till puberty, and even during adult life (1). Has irradiation permanently divested the presumably undifferentiated cells of the germinal epithelium of the power of transforming into germ cells, or has it destroyed the mechanism of the environmental stimuli that brings about this transformation ?

The effects observed on the follicles may not be direct, but may be entirely due to the destruction of the oocytes within them. This is not so, however, with the corpora lutea atretica, formed after irradiation from the atretic follicles, because they become persistent. The functional corpora lutea vera of the adult animal exhibit a similar persistence when subjected to irradiation. Normally luteal tissue of any kind does not persist for long in the mouse, and never more than the three weeks of lactation.

The abnormal giant cells, etc., of the luteal-like tissue of the first proliferation are, probably, also caused directly by the rays. Mitoses are by no means inhibited in the tissues of the ovary as a whole and, on the contrary, appear to be stimulated.

V.—Summary.

1. The ovaries of six mice X-rayed *in utero* and of thirty X-rayed at birth are described.

2. The results confirm those obtained with mice X-rayed at three weeks old (2). The degeneration of the oocytes and follicles is followed by two successive proliferations from the germinal epithelium in the form of cords.

3. Some of the degenerating follicles produce cysts or corpora lutea atretica. The latter may persist indefinitely but have no effect on the œstrous cycle.

4. Of the animals irradiated at birth 24 were allowed to become adult. The cords of the first proliferation constituted the bulk of all the ovaries, and those of the second proliferation were only found in 11 of the cases. Follicles were completely absent in all but one case.

5. These 24 animals are divided into three groups. (*a*) The cells of the first proliferation are small, shrunken and vacuolated. The œstrous cycles are irregular and in some cases prolonged vaginal cornification occurred. (*b*) The cells of the first proliferation are large, healthy and glandular in character, and somewhat resemble luteal tissue. The œstrous cycles were normal and regular. (*c*) The cells of the first proliferation very closely resemble luteal tissue. The œstrous cycle was absent or had ceased for at least 36 days previous to being killed.

6. It is concluded that the cells of the first proliferation are mainly responsible for the production of œstrin and the regulation of the œstrous cycle. The production of œstrin appears to stop when the differentiation into luteal-like cells attains a certain degree.

Our thanks are due to Prof. J. P. Hill, F.R.S., and to Dr. F. H. A. Marshall, F.R.S., for their interest and advice, and also to Mr. F. Melville for carrying out the irradiations.

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DESCRIPTION OF PLATE FIGURES.

GUIDE LETTERS.—*T.A.*, Tunica albuginea. *G.E.*, Germinal epithelium. *B.V.*, Blood vessel. *F.C.*, Cavity representing old follicle. *M.R.*, Loose medullary region. *E.C.*, Epithelial canal. *C.*1, Cord of first proliferation. *C.2a*, Intermediate type cord of second proliferation. *C.2b*, Cord of second proliferation. *S.T.*, Old interfollicular tissue. *N.F.*, Follicle. *G.C.*, Giant cell. *L.A.*, Corpus luteum atreticum.

PLATE 7.

FIG. 1.—Ovary of BX 69, showing the cords of the first proliferation with enlarged vacuolated cells. × 66.

FIG. 2.—Ovary of FSX 2 ♀, showing follicles, cavities representing old follicles and twenty-four corpora lutea atretica. × 36.

FIG. 3.—Ovary of BX 13, showing epithelial canals and very luteal-like tissue of the first proliferation, characteristic of Group III. × 66.

FIG. 4.—Ovary of BX 75, showing somewhat luteal-like tissue of the first proliferation, characteristic of Group II, and cords of the second proliferation. × 50.

FIG. 5.—Ovary of normal new-born mouse for comparison. × 133.

FIG. 6.—Ovary of BX 77, showing the small shrunken-celled tissue of the first proliferation, characteristic of Group I. × 47.

PLATE 8.

FIG. 1.—Ovary of BX 42, showing two growing follicles and the absence of small oocytes. The first proliferation has just begun to form. × 166.

FIG. 2.—Portion of the ovary of BX 51, showing giant cells in the very luteal-like tissue of the first proliferation. × 320.

FIG. 3.—Portion of Plate 7, fig. 4, at higher magnification, showing cords of the first proliferation and cords of the second proliferation forming from the germinal epithelium. × 290.

FIG. 4.—Ovary of FPX 6 ♀ *a*, showing tissue, which is probably the first proliferation and epithelial canals. × 62.

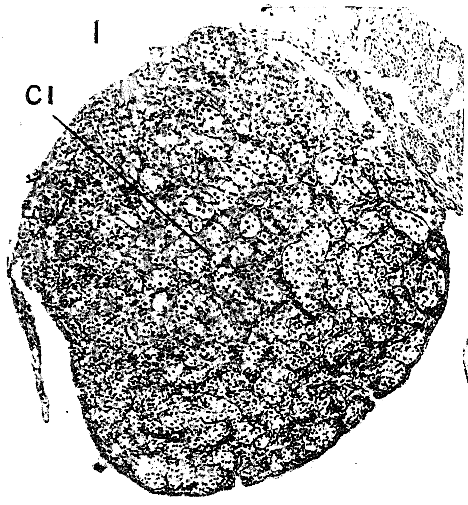
PLATE 9.

FIG. 1.—Portion of Plate 7, fig. 3, at higher magnification, showing the extremely luteal-like cells of the first proliferation and epithelial canals. × 275.

FIG. 2.—Portion of Plate 7, fig. 1, at higher magnification, showing the enlarged vacuolated cells of the first proliferation. × 275.

FIG. 3.—Portion of Plate 7, fig. 6, at higher magnification, showing the small shrunken cells of the first proliferation. × 290.

We are indebted to Mr. F. J. Pittock for the photomicrographs reproduced in this paper.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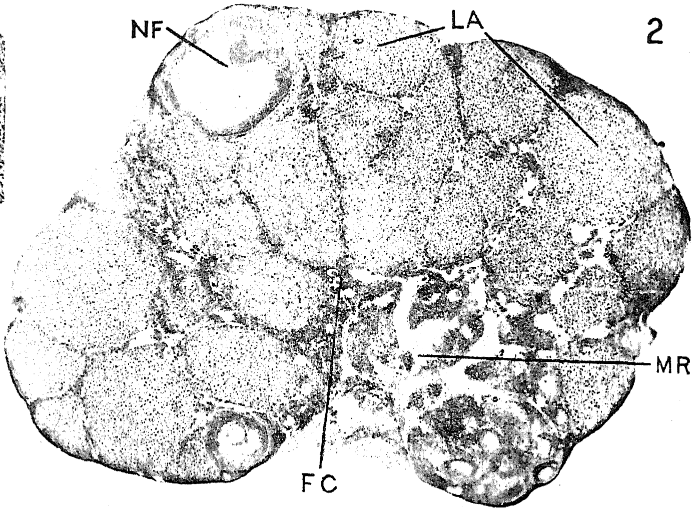
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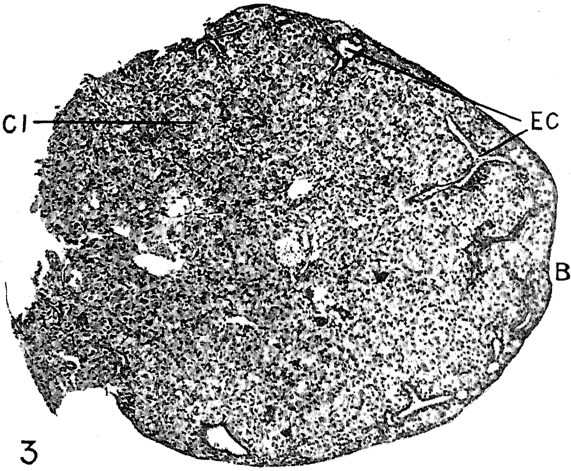
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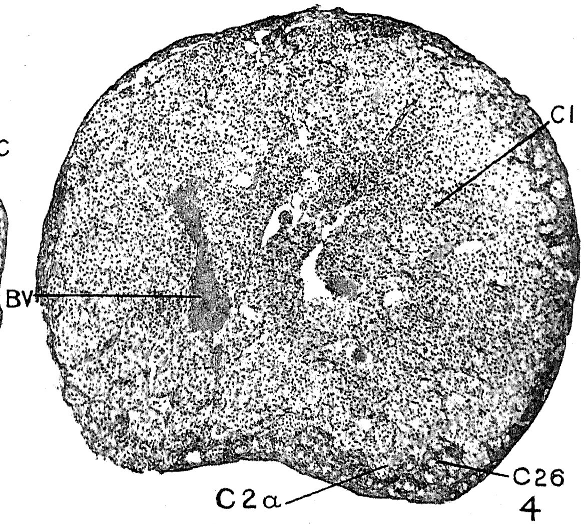
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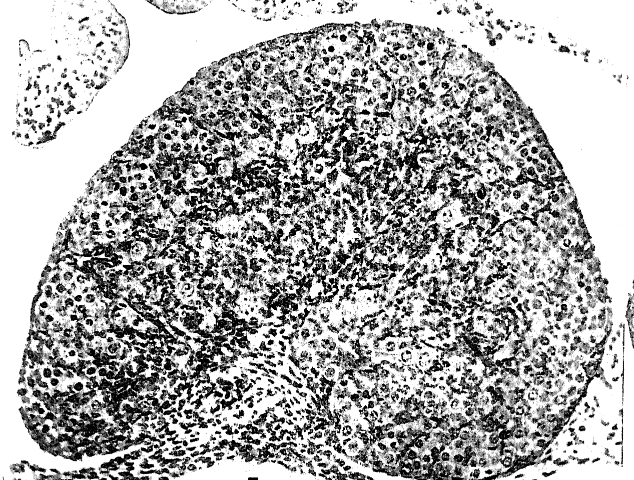
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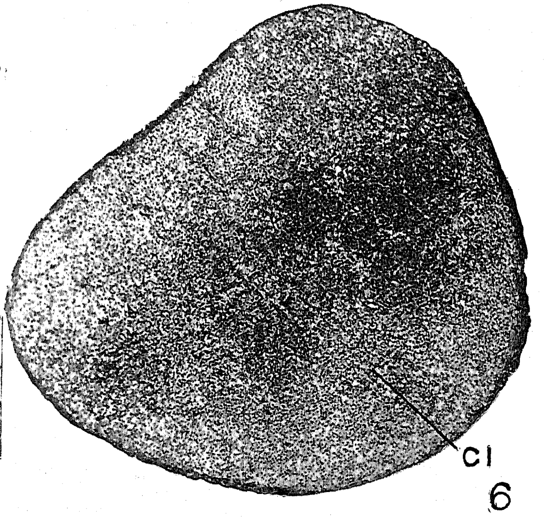
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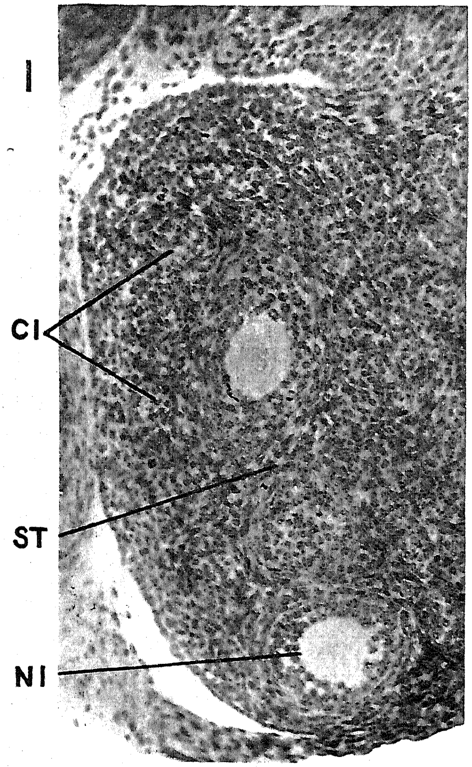
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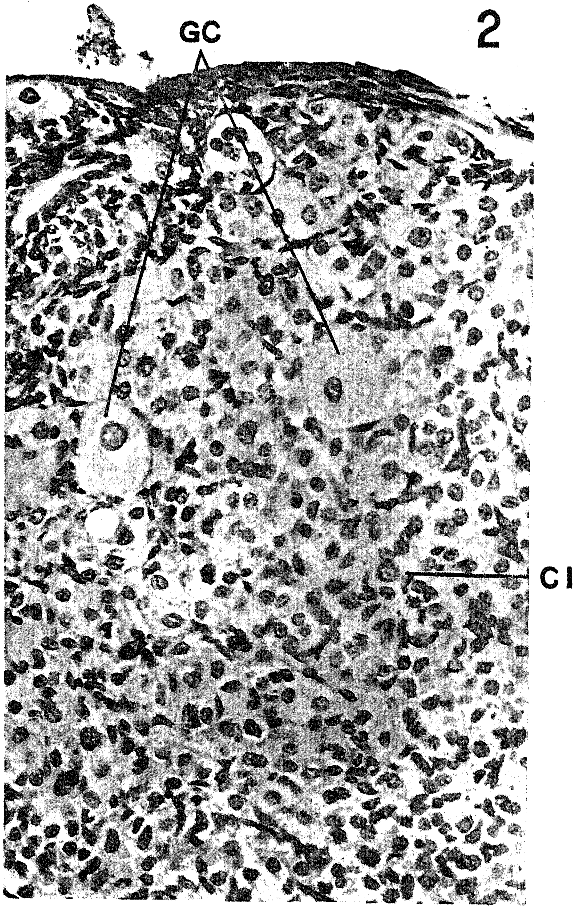
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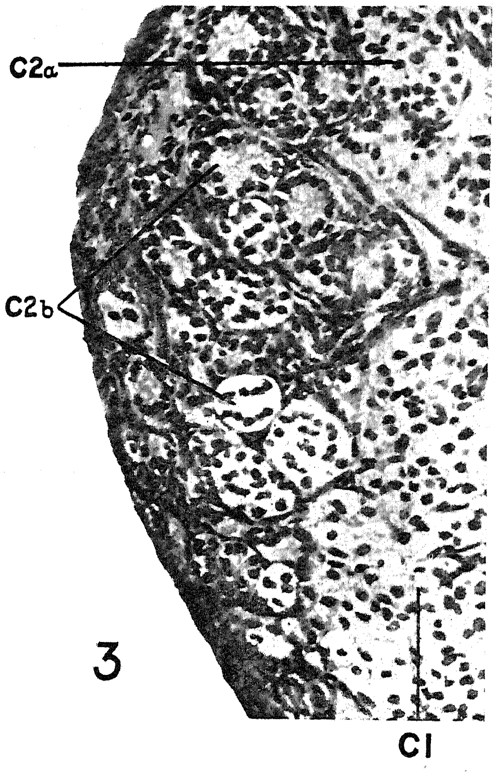
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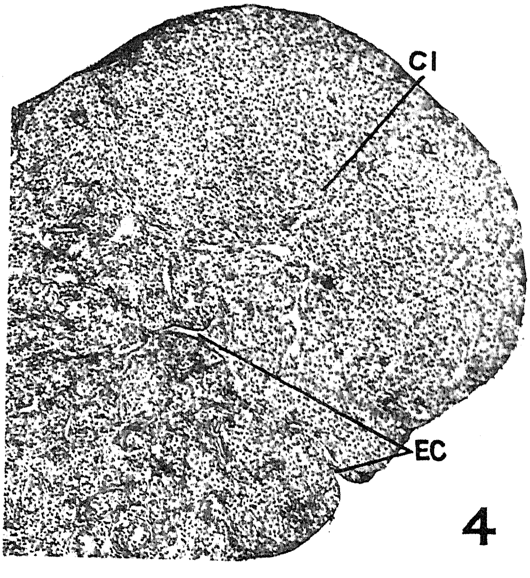
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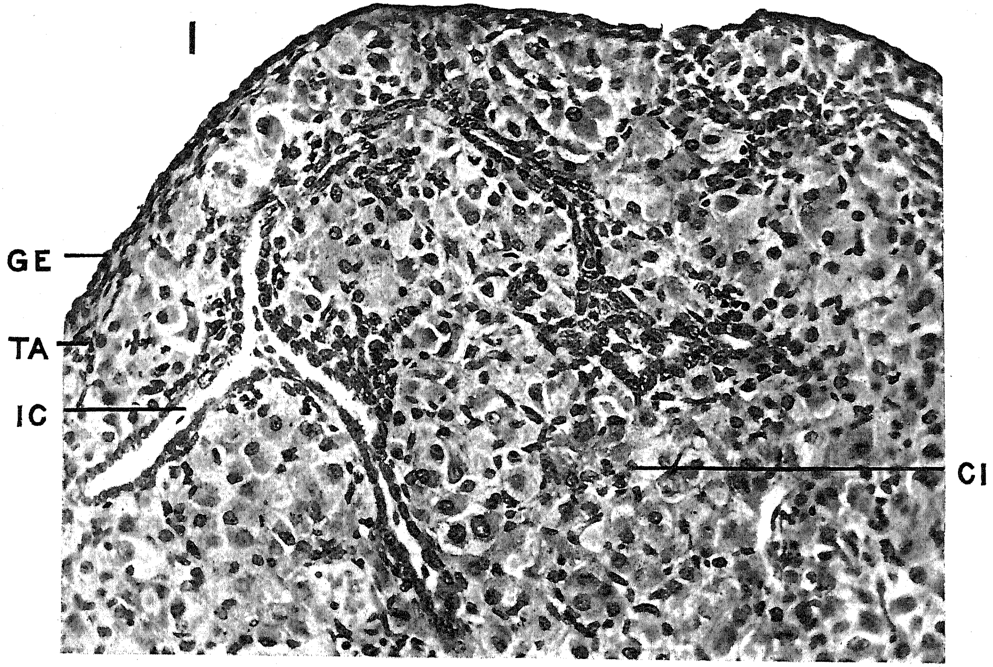

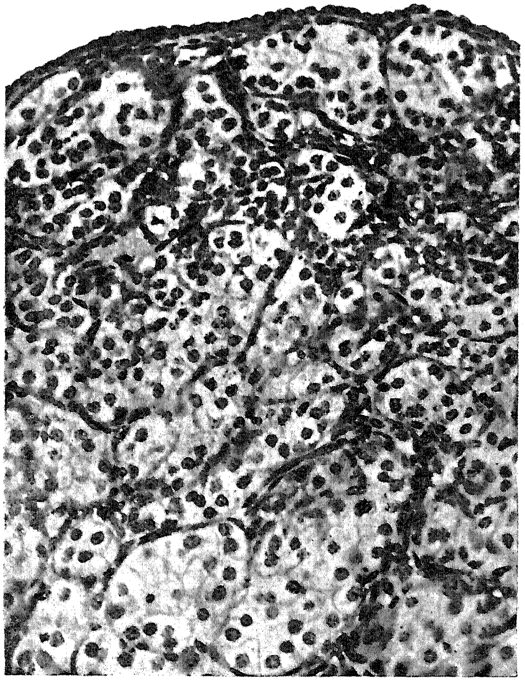

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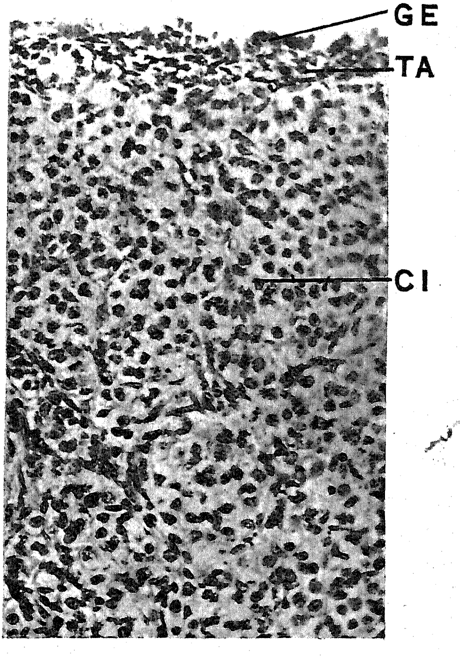

3

Experiments on the Egg-laying Instincts of the Sawfly, Pontania salicis *Christ., and their Bearing on the Inheritance of Acquired Characters; with some Remarks on a New Principle in Evolution.*

By J. W. Heslop Harrison, D.Sc.

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I. *Introductory.*

In the course of genetical investigations (Harrison, 1920) in the lepidopterous genus *Oporabia* I conceived the idea that the inherited instinct of *Oporabia filigrammaria* HS. to deposit its eggs on heather (*Calluna vulgaris*) was the direct result of long-continued isolation on treeless heather-clad moorlands, involving a compulsory diet of that plant; in other words, I felt that the development of the instinct and its inheritance were Lamarckian effects. To test these views experimentally, by attempting a transference in the food habits of other insects, seemed far from difficult, and search was forthwith made for material easy to manipulate in captivity.

No forms appeared more adapted for the purpose than the Geometrid moths *Lycia hirtaria* Cl. and *Phigalia pedaria* F., which I had employed in former experiments. However, the technique adopted, at any rate as applied to these insects, proved unsatisfactory and had therefore to be modified. Unexpected difficulties, not in securing oviposition in a state of semicaptivity but in its taking place in a natural manner on the food plant, were immediately encountered.

Fruitless as this preliminary work was, it nevertheless served to show that any research along these lines could only be conducted under conditions approximating more or less closely to those obtaining in nature. One of these species, *Lycia hirtaria*, was therefore transferred to the garden and allowed to feed up as larva on privet (*Ligustrum vulgare*) and to pupate naturally in the soil where it was to be left for hibernation. This course was persisted in for three years, but on the expiry of that period the species had failed to establish itself and had vanished.

Clearly the use of species much less open to the attack of birds was indicated, and such insects were discovered in the gall-making species of sawfly (*Hymenoptera Tenthredinoidea*) belonging to the genus *Pontania*. Since the eggs of these species are laid within willow leaves, and the galls surround the larvæ from the moment they hatch, they possess the additional advantage of being brought into very intimate contact with the tissues of their food plant quite independently of its being ingested as food.

Of that genus *Pontania salicis* Christ., *P. vesicator* Bremi and *P. proxima* Lepel. appeared, after a careful study of their behaviour in the field, to be pre-eminently suitable for the work. However, the common occurrence of the last-named species in a wild condition in this neighbourhood made the danger of contamination of the experimental cultures from wild stock so great that its use was not to be considered.

Furthermore, preliminary tests carried out with the other two revealed serious practical difficulties in the case of *Pontania vesicator* which, owing to its predilections for sub-alpine localities, proved to be a very awkward form to establish in this area. (Birtley, North Durham.) Thus only the one species, *Pontania salicis,* remained, and it, fortunately, provided me with ideal material for the research.

The species of *Pontania,* like those of the Cynipid genus *Rhodites,* discussed from the experimental standpoint in previous papers (Harrison, 1922, 1926), are remarkable in their choice of food plants. All feed on willows (*Salix* spp.), but each colony possesses its own special species of *Salix* as food plant to the exclusion of all other species growing near by, even when the latter are favoured elsewhere.

Pontania salicis, for example, is recorded by Houard (1908–13) from *Salix acutifolia, S. alba, S. triandra, S. arbuscula, S. aurita, S. babylonica, S. caesia, S. cinerea, S. daphnoides, S. daphnoides* × *S. repens, S. depressa, S. fragilis, S. glabra, S. hastata, S. helix, S. helvetica, S. incana, S. lapponum, S. Myrsinites, S. Andersoniana, S. pentandra, S. phylicifolia, S. puberula, S. purpurea, S. repens, S. reticulata, S. rubra, S. salviifolia* and *S. viminalis* ; nevertheless, in Durham and Northumberland, in Upper Teesdale, the same species is restricted to *S. phylicifolia,* at the Sneap (Derwent Valley) and in Prestwick Carr to *S. Andersoniana,* on Waldridge Fell to *S. aurita,* on the Tyne, westward from Newcastle as far as I have explored, to *S. purpurea,* on the Durham coast to *S. repens,** and so with many other stations investigated by me.

Similarly, abroad, at Cauterets in the French Pyrenees, and near Aja, Spain, the colonies noted confined themselves to *S. purpurea* in spite of the presence of other *Salices* quite suitable as food plants.

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* This colony is extremely striking ; there *S. repens,* in a most unusual manner, grows luxuriantly intermingled with *S. Andersoniana* to such an extent that hybrids abound. Nevertheless, no galls of *Pontania salicis* are to be found on *S. Andersoniana,* whilst those occurring casually on the hybrids rarely, if ever, develop fully, just as happens in the case of *Rhodites rosæ* from *Rosa mollis* colonies when transferred to *R. canina* (agg.) (Harrison, 1926).

Again, *Pontania proxima,* in one station in this area, is only to be found on *S. cinerea,* whilst a little more than three miles away, although that shrub abounds, the only species attacked are those of the *alba-fragilis* group. More curiously still, at Nuria in Spain, the insect can be collected only from *Salix herbacea.* Nor is this peculiarity restricted to the Palæarctic species, for I noted parallel facts in connection with *Pontania pomum** Walsh in Canada; near Meadowvale in Ontario it favoured *S. cordata,* whilst at Glacier, British Columbia, *S. scouleriana* alone bore its galls.

In this restriction of individual colonies to a single *Salix* species as food plant was offered an excellent opportunity of determining whether long continued oviposition on that special plant had fixed the food habit germinally. Thus the problem proposed for solution could be approached in two distinct ways, one capable of affording absolute proof of the validity of the Lamarckian position, and the other yielding evidence of prime importance leading to the same conclusion.

The two lines of attack, both of which were adopted, were these:

(1) Attempts could be made, by removing a strain of the insect chosen for the work from the food plant to which it had been accustomed, to a habitat in which a wholly different species of *Salix* grew, to compel it to take to that species. If it proved wholly or partially acceptable, the new colony could be allowed to develop on it for some years. At the end of that stage the hereditability of the new food habit could be tested by giving the insect access once more to its original food plant.

(2) In much the same way, a strain could be taken to a station in which many species of *Salix*, including its own normal food plant, were available for oviposition. By direct observation of the species upon which gall development took place deductions immediately applicable to the solution of the problem in hand could be made.

Although the first-named experiment was commenced in 1919, and the second in 1923, it is more convenient to describe the last set first.

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* I had intended to experiment on a large scale with this species and *Pontania pisum* Walsh, and took great numbers of galls in Canada for the purpose. However, the failure of the insects to emerge when the willow leaves were in the optimum condition for oviposition made work in this climate with them impossible.

II. *Experiments designed to test the Germinal Fixation of the Food Habit in Individual Colonies.*

For obvious reasons, this portion of the work could not be conducted in a natural habitat; nor was it possible to carry it out in the garden I had at my disposal in 1919. In the first place, this was to a considerable extent congested with the wild roses devoted to the parallel work with *Rhodites rosæ,* and, in the second, it was impossible to let it run wild so that satisfactory cover in the way of undergrowth might be accessible to the *Pontania* larvæ for cocoon-spinning purposes.

However, in 1921, I secured a large garden entirely satisfying my requirements. On its northern boundary it broke into natural woodland fringed by a rank growth of *Rubus fruticosus* (agg.), *Rubus idæus, Cratægus Oxyacantha* and various other plants. To this tangle I added very materially by planting birches, alders, various wild roses and, in addition, a very wide selection of *Salices.* These were allowed until 1923 to establish themselves, in which year there were growing in the garden the following species of sallows and willows:—

SECTION PENTANDRÆ: *Salix pentandra L., S. lucida* Muhl.
SECTION TRIANDRÆ: *S. triandra* L.
SECTION FRAGILES: *S. fragilis* L.
SECTION ALBÆ: *S. alba* L.
SECTION RETUSÆ: *S. herbacea* L.
SECTION SERICEÆ: *S. glauca* L.
SECTION PRUINOSÆ: *S. daphnoides* Vill.
SECTION VIMINALES: *S. viminalis* L., *S. purpurea* × *S. viminalis* (= *S. rubra* Huds.).
SECTION HASTATÆ: *S. cordata* Muhl.
SECTION PHYLICIFOLIÆ: *S. phylicifolia* L., *S. Andersoniana* Sm.
SECTION CAPREÆ: *S. Caprea* L., *S. aurita* L., *S. cinerea* L., *S. aurita* × *S. repens, S. cinerea* × *S. repens, S. cinerea* × *S. purpurea.*
SECTION REPENTES: *S. repens* L.
SECTION PURPUREÆ: *S. purpurea* L., *S. cæsia* Vill.

All of these were then in a very flourishing condition and easily reached by female sawflies seeking to deposit their ova.

In the autumn of the same year, therefore, several thousands of galls of *Pontania salicis,* taken, without exception, from *S. phylicifolia,* were collected in Upper Teesdale. These galls were placed in 10-inch plant pots, half full of baked coconut fibre, in which the larvæ could spin their cocoons, the mouth of the pot being covered with linen tied securely by thin string. Curiously enough these measures, so satisfactory generally, proved utterly unsuitable here, for the larvæ, instead of burying themselves for pupation, came upward to spin their cocoons between the linen and the rim of the pot—a course which could only end in their destruction. To obviate this difficulty, crushed crinkled paper was placed on the top of the galls to which the larvæ could retire, and hundreds of cocoons were spun in the folds of the paper.

In October the pots were placed outside, about 26 yards from the willow thicket under a light wooden protection facing north (*i.e.*, toward the willows) and open on that side to the weather.

Almost simultaneously with these preparations, supplies of galls of the same sawfly were collected from *Salix purpurea* growing in the Hexham area, and subjected to the same treatment.

In the spring of 1924 the coverings were removed from the plant pots so that the emerging sawflies were at liberty to fly for oviposition just where they desired. The insects from Upper Teesdale began to appear first, and in the first and second weeks in May numbers were to be seen darting about the garden in the sunshine, whilst pairs *in copulâ* were of free occurrence on the willow leaves.

Very early indeed, gall development became observable, and as soon as the numbers showed no increase a census was taken. The colonisation of the garden having been perfectly successful, the insects were allowed to breed without interference on my part in 1925 and 1926, when further counts were made, the results of which, with those of 1924, appear in the appended table.

The meaning of the figures cannot be mistaken; the ovipositing females, except in certain cases in which the hybrid *S. purpurea* × *S. viminalis* has been selected, have utilised *S. phylicifolia* and *S. Andersoniana*, two species so closely allied that they are not only placed by all competent salicologists in the Section Phylicifoliæ, but, in addition, are regarded by such an excellent worker as Buchanan White (1891) as being specifically the same.* Such a striking result is in entire harmony with those secured in the *Rhodites* work of 1922 and 1926.

However, one important fact stands out, and that is that the insects, although derived from a *S. phylicifolia* habitat, evinced in 1925 a marked preference for *S. Andersoniana* to the comparative neglect of *S. phylicifolia*. The excess of galls on *S. Andersoniana* in the first year was very possibly due to the position

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* This position, independently of obvious differences in the plants in the field, cannot be maintained in face of the discovery (Blackburn and Harrison, 1924) that the haploid chromosome number of *S. phylicifolia* is 44 and of *S. Andersoniana* 57.

Table I.

Salix species or hybrid.	Galls from the *Salix phylicifolia* strain.			Galls from the *Salix purpurea* strain.
	1924.	1925.	1926.	1924.
S. pentandra L.	—	—	—	—
S. lucida Muhl.	—	—	—	—
S. triandra L.	—	—	—	—
S. fragilis L.	—	—	—	—
S. alba L.	—	—	—	—
S. herbacea L.	—	—	—	—
S. glauca L.	—	—	—	—
S. daphnoides Vill.	—	—	—	—
S. viminalis L.	—	—	—	—
S. purpurea × *S. viminalis*	43, all aborting	3, all aborting	7	—
S. cordata Muhl.	—	—	—	—
S. phylicifolia L.	237	6	60	—
S. Andersoniana Sm.	315	245	572	—
S. Caprea L.	—	—	—	—
S. aurita L.	—	—	—	—
S. cinerea L.	—	—	—	—
S. aurita × *S. repens*	—	—	—	—
S. cinerea × *S. repens*	—	—	—	—
S. cinerea × *S. purpurea*	—	—	—	—
S. repens L.	—	—	—	—
S. purpurea L.	—	—	—	268
S. cæsia Vill.	—	—	—	—

of several of the bushes of that species, a little in front of the first *S. phylicifolia*, but this could not explain the state of affairs in 1925. Then it seemed as if a tendency to select *S. Andersoniana* had developed as a result of the circumstances noted in 1924, and had been inherited.

To test this possibility, in the late autumn of 1925, a second small plantation of *S. Andersoniana* was made about 15 yards from the main groups and shut off from all save two hybrid *S. purpurea* × *S. viminalis* by a belt of roses, brooms, birches and alders. At intervals between this plantation and the older one were inserted " trap " examples of *S. phylicifolia*, of which species additional " trap " specimens were scattered irregularly over the garden.

In the following year, although the female sawflies must have flown over these isolated *S. phylicifolia* bushes, no gall formation was detected on them, whilst on the far more remote, additional *S. Andersoniana* no fewer than 139 galls were counted. The meaning of these results is obvious, but is not stressed here in view of those secured in the experiments described in section III below.

It will have been noted that in 1924 quite an appreciable number of ova were deposited on the *S. purpurea* × *S. viminalis* plants. Not a single one of these yielded a full-sized gall, all aborting in a very immature stage, and the larvæ, if any,* dying. In 1925 careful examination leaf by leaf disclosed only 3 galls on that hybrid, although its various representatives, except for certain examples of *S. viminalis*, were the best grown shrubs in the garden. However, in 1926, 7 galls on that plant reached maturity, and the larvæ they contained spun up quite safely.

The case of the contingent secured from *S. purpurea* must now be considered. These emerged from the pupæ early in June, 1924, and proceeded to oviposit at a time when gall formation in the case of the *S. phylicifolia* series was well advanced. Very soon counts were possible, when it was found that, without exception, all the galls, produced in some numbers, were upon *S. purpurea*, although some of the specimens of that species were farthest away of all from the shelter containing the plant pots.

This result is in perfect agreement with the outcome of the *S. phylicifolia* work. There can be no doubt but that the instincts guiding the *Pontania* female in the choice of food plant are germinally fixed.

At this point it ought to be stated that, as there were present in the garden in 1924 strains of *Pontania salicis* from two colonies differing in their food plants, if the work was to continue one must be removed. For reasons, which will be obvious from what has just been written, it was decided to extirpate the *S. purpurea* race, which was done by having every gall collected from the plants in the September of that year. This collection must have been effective for no galls have since occurred on *S. purpurea*.

III. *Experiments designed to develop, if possible, a new Food Plant Instinct and to test if that Instinct is inherited.*

As previously explained, no garden was available in 1919 for work demanding an approximation to natural conditions. Fortunately, however, I had permission to visit a very secluded piece of waste land upon which grew a colony of *S. rubra*—a hybrid between *S. purpurea* and *S. viminalis*.

In the choice of a race of *Pontania salicis* with which to commence I was influenced by the necessity for avoiding, if possible, *S. purpurea* strains, since the plant with which experimentation was planned was a hybrid of that species. Unless, therefore, I could reach the more inaccessible points of Upper Teesdale in September or early October no other option was left but to choose *S. Andersoniana* feeding insects or those drawn from *S. repens* on the coast. Ultimately

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* Gall development in the Tenthredinoidea commences with the laying of the egg not with the hatching of the larva.

I determined to avail myself of *S. Andersoniana* forms from the "Sneap," a curious peninsula, not far from Muggleswick, almost surrounded by the River Derwent.

Large numbers of gall were collected, and the larvæ they yielded allowed to pupate in plant pots in the usual manner. These instead of being placed for the winter near the thicket in which it was desired to establish them, were retained in the garden until May, 1920, when they were transferred to the experimental plot and concealed as well as possible there. Owing to their remote situation I could not make daily visits to these plants, as I was wont to do with the others, but had to content myself with scrutinising them at longer intervals.

Early in June I was delighted to find incipient galls, but my hopes of the successful foundation of a colony were almost destroyed in July, for in that month almost every gall showed signs of abortion. Nevertheless, in the end, many more than I had dared to hope for reached maturity.

In the succeeding year (1921) the insects issuing from these galls were reinforced by a fresh contingent introduced from the *S. Andersoniana* area and released in the same way. Again gall development proceeded, but once more abortion was a common occurrence. Still, enough well-grown galls were present in September to justify my leaving the insects to take care of themselves.

In 1922, in spite of the failure of some, quite a good percentage of the galls matured so that the colony could now be regarded as fully established. It was, however, left alone until the winter of 1923–24, when six examples of *S. Andersoniana* were planted near, and in, the experimental thicket.

Owing to my absence in Canada in 1924, and to other mishaps, I was unable to examine the colony until 1925, when I found that not only was it in a flourishing state but that the *S. Andersoniana* plants were quite free from galls. In other words the acquired habit of oviposition on *S. purpurea* × *S. viminalis* has been germinally fixed.

This year (1926) the conditions were precisely the same; there was an abundance of galls on the hybrid but not a single* one on the *S. Andersoniana*.

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* The failure to secure a single gall from the *S. Andersoniana* seems so remarkable to me that I have collected every gall I could find from this colony for further experiments. One half of these I intend to attempt to transfer to a *Salix repens* station on the Little Fell here, quite free from any hymenopterous gall-making insects, and the other half I shall endeavour to throw back on *S. Andersoniana*, which I am getting ready for their reception in the old garden in which the first *Rhodites* work was carried out.

We are thus dealing with an incontrovertible case of the inheritance of acquired characters.

IV. *Concluding Remarks.*

From the above experiments three very important deductions can at once be made :—

(1) That one and the same species may be broken into local races each with its own special food plant not necessarily the same as that accepted in adjoining stations.

(2) That an acquired character, *e.g.*, egg-laying instinct, can be inherited.

(3) That therefore the development of such races as are mentioned under (1) may take place at any time.

Lately, in conjunction with Garrett (Harrison and Garrett, 1926) and alone (Harrison, 1926), I have described researches of far-reaching importance in which certain lepidopterous species *Selenia bilunaria* Esp., *S. tetralunaria* Hufn., *Tephrosia bistortata* Goeze, and *T. crepuscularia* Hb., instead of receiving their natural food were supplied with foliage artificially impregnated with chemical compounds quite foreign to their normal diet. As a result, after a few generations of this treatment, there developed in the cultures melanic forms differing widely in their characters from those proper to the species employed. Furthermore, the melanism so induced proved to be inherited in a normal Mendelian fashion so that if two insects carrying it in a homozygous condition were mated together they bred quite true.

It cannot be denied that the differences between species, plant and animal alike, are chemical in their nature. Hence, if insects specialised in their food plants are transferred by any accidental circumstance to another plant, and are able to maintain themselves thereon, they are subjected to chemical action, due to the ingested food, differing from that proper to them ; they are thus in the position of the Geometrid moths used in our experimental cultures. Therefore, they are liable to react in the same way as the experimental moths with the consequent appearance of new characters. In other words, they are being forced along an evolutionary path away from the parent species.

If their reaction to the changed food plant parallels that of the lepidopterous insects enumerated above they should show colour differences. And such, indeed, is the case with the Pontanias with which we have been immediately concerned. The race from *Salix phylicifolia* is bright brown, and differs greatly from the dingy insect from *S. purpurea* and likewise from that frequenting *S. Andersoniana.*

Again, although their inheritance has not been worked out, morphological and other differences, in addition to changes in wing colouration, appeared in the *Selenia bilunaria* cultures, wing shape, duration of larval life and other characters being involved, and much the same differences are to be expected in the *Pontania* races. These latter, therefore, isolated from one another in respect to food plant, with characters diverging from those of the central type from which they are derived, can only be regarded as species in the making. In other words, we see the evolution of species taking place, not on the basis of the older theories, but on an entirely new principle.

If such views be correct, and there appears to be no other interpretation possible either of the experimental or of the observed facts, we ought to find, more especially in the phytophagous Insecta, groups of closely allied species, with distinct food plants, but with these food plants so associated in nature that the passage of larvæ from one to the other, no matter how brought about, is a very simple matter.

In a most significant way, many such species are known even within the bounds of our own limited fauna. For instance, marshy thickets composed of an indiscriminate mixture of alders (*Alnus rotundifolia*) and sallows (*Salix aurita, S. cinerea et S. Caprea*) are of frequent occurrence in Britain. Thus accidental transferences of larvæ from alder to sallow, or *vice versa*, by the agency of the wind, errors in oviposition, and otherwise, must often take place. On these we have several pairs of species such as those postulated. Amongst them the Notodontid moths *Notodonta dromedarius* L. and *Cerura bicuspis* Bkh. on alder correspond to *Notodonta ziczac* L. and *Cerura furcula* L. on sallow, the Geometrid *Ypsipetes trifasciata* Bkh. to *Y. ruberata* Frr., the Sesiid *Sesia sphecíformis* Gern. to *S. formiciformis* Esp., and so on.

Nor is this matter of allied pairs only to be noted in the lepidoptera ; in the Psyllidæ (Hemiptera Homoptera) the alder species *Psylla försteri* Flor. can be compared with the sallow *P. salicicola* Först, in the Hymenoptera (Tenthredinoidea) the alder *Cimbex connata* Schr. balances the sallow *C. lutea* L., in the Cecidomyidæ (Diptera) the alder *Perrisia alni* F. Löw corresponds to *P. auritæ* Rübs., and, if appeal be made to phytophagous groups other than the Insecta, the Eriophyid mite *Eriophyes lævis* Nal. from alder can be set alongside *E. tetanothrix* Nal. from sallow.

Pursuing the matter further we find other groups of plants ecologically connected. *Saxifraga aizoides* clashes in many stations with *Calluna vulgaris* ; attached to the former is *Larentia flavicinctata* Hb. and to the latter *L. cæsiata* Lang. In oakwoods the undergrowth frequently includes *Lonicera*

Periclymenum and *Rubus fruticosus* (agg.), the oak harbouring the Aleyrodid (Hemiptera Homoptera) *Aleyrodes quercus* Sign., the honeysuckle *A. loniceræ* Walker, and the bramble *A. rubi* Sign. In many sub-alpine birchwoods where birch and aspen flourish together, we find the moth *Pheosia dictæoides* Esp. on birch and *P. tremula* Cl. on aspen—and so the list could be lengthened indefinitely.

Clearly the facts in nature support fully the principle I have enunciated. But its validity does not end with the instances already set out. If chemical differences due to soil affect plants similarly then we see opened out an explanation, not only of the evolution of the plant species in nature, but also of their breaking into new forms when brought under cultivation.

Similarly, parallel explanations would suffice to account for variation in groups of animals differing widely from the Insecta, as well as for that observed when wild animals are domesticated. To be exact, these views put variation (and therefore evolutionary action) on a vastly different plane from what it has previously occupied; now, a definite reason for its occurrence has been offered, supported by experimental evidence of its truth in the case of certain phases of variation.

Moreover, its value does not end here. Clearly such evolution as is pictured at work here is not of the Lamarckian order; most likely the influences at work act directly and simultaneously on soma and germ alike, or even on germplasm alone, and, indeed, the latter view obtains concrete support from the *Selenia bilunaria* work. If, however, the germplasm can be influenced in this matter, the step to admitting the truth of Lamarckian doctrine is but slight, and one almost involuntarily to be made, when it is realised how easy it would be for chemical action registered in the soma to be communicated by some method, whether by means of the blood-stream or otherwise, to the germplasm.

Add to this the fact that a direct demonstration of a genuine Lamarckian effect has been made, and one is forced to admit that Lamarckism itself has received very powerful support, both directly and indirectly, from the work described above.

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*Experiments on the Physiology and Genetics of the Smut Fungi. —Hyphal-Fusion.**

By Sydney Dickinson, Rothamsted Experimental Station, Harpenden.

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[Plate 10.]

I. *Introduction.*

In recent years attention has been devoted to the study of the Smut Fungi, and their cytology is now fairly well known. The results of previous observations on the two Smuts used in the following experiments may be briefly summarised :—The Chlamydospore in both species is uni-nucleate, and on germination a promycelium is formed into which the nucleus passes. There it divides into two, and the two daughter nuclei pass, one to each end of the promycelium, and there divide, so forming four nuclei between which cross walls are then formed (see text-fig. 1, A–E, p. 130). After this stage the behaviour is governed largely by the environmental conditions, either one or more sporidia are budded off from each segment or clamp connections are formed between pairs of segments. (Text-fig. 1, F–G.) No linear order is apparent in these unions, all possible combinations having been observed ; and the nucleus and cytoplasm of one segment passes into the other through the clamp connection.

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* This work was carried out in the Department of Mycology, Rothamsted Experimental Station, Harpenden, Herts, with the aid of a grant from the Ministry of Agriculture and Fisheries.

Again, no crossing or changing over in position of the nuclei in the unsegmented promycelium has been seen. It has been presumed therefore that the nuclei in the segments, if numbered from the apex, 1, 2, 3, 4, are in pairs, 1 and 2 being the product of one nucleus, 3 and 4 the product of the other. This has been corroborated in the present work.

Kniep (1) by isolating sporidia at random from a mass of germinating chlamydospores showed that such sporidia could be arranged in two groups, the members of the first, called "A," conjugating with those of the second called "B.". No sporidium would conjugate with another of its own group. The numbers in each group were approximately equal, and, from this and the facts already cited, it was deduced that the segregation of "sex" takes place in the second nuclear division in the promycelium.

That two "sexes" are present in many of the Smuts has been shown by Bauch (2); and in one fungus, *Ustilago longissima*, he has described a tri-sexual condition. The sporidia, isolated at random from a mass of germinating chlamydospores of this species, could be arranged in three groups, the members of which, while not conjugating within the group to which they belonged, would conjugate with any from the other two groups.

Kniep (3) has observed conjugation between the germ tubes of sporidia from a number of species of Smuts, and this has also been seen during the present work. By adding conidia from a culture of known "sex" to germinating chlamydospores and observing the conjugations which take place, Kniep (3) has also demonstrated that there is no regularity of position of the "sexes" in the promycelium.

Previous authors have described clamp connections between segments of the promycelium and conjugation between germ tubes of sporidia. In the present work union between the hyphæ of two mycelia is described, analogous to the reactions of the + and — strains of the Phycomycetes (Blakeslee, 4). This behaviour has been used for the determination of "sex." The Smuts used in the experiments were Covered Smut of Oats, *Ustilago levis*, and Covered Smut of Barley, *Ustilago Hordei.*

II. *Technique.*

Chlamydospores were placed on 1 per cent. beef extract agar films on cover slips kept in moist Petri dishes and left in incubators for 12 or more hours at various temperatures. As soon as the spores had germinated and each segment of the promycelium had produced one sporidium, these four sporidia were isolated in turn by means of the "Dickinson Isolator" (5) and transferred to beef extract agar slopes in test tubes. Each tube was given a fractional number, whose numerator signified the parent chlamydospore, and whose denominator signified the position of the segment on the promycelium from which the particular sporidium had been removed; the segments were numbered from the apex 1, 2, 3, 4. Thus CSOK52/1 means Covered Smut of Oats, Kimpton (specimen and origin); 52 the number of the chlamydospore, and /1 the sporidium isolated from the apical segment of the promycelium.

The cytological observations were made on material which had been germinated or grown on nutrient agar on cover slips or in Petri dishes, and which had been affixed to microscope slides in the following manner:—A thin slice of agar, on the surface of which the mycelium was growing, was cut out of the Petri dish with a scalpel. The mycelium was killed by exposure to osmic acid vapour for about five minutes. The slice was then allowed to dry for 15–30 minutes, exposed to the air of the room, and afterwards inverted on to a microscope slide smeared with fresh egg albumen. The slide was immediately put into strong Flemming's solution for half an hour. After washing in distilled water the specimen was decolorised with hydrogen peroxide and then stained. The stain finally adopted was Heidenhain's hæmatoxylin. After staining the agar was lifted off with a scalpel, this requiring considerable care lest the mycelium be removed at the same time; in delicate cases the agar was removed with the aid of the Isolator. The hyphæ were thus left fastened to the slide in the same relative positions as they had occupied when growing. After de-staining, the transference from water to alcohol was carried out gradually (5 per cent. stages) to prevent shrinkage of the hyphal contents.

Where special stages of development were required, likely hyphæ or conidia were moved from the main culture mass to a fresh part of the film, and there watched; or put into contact with other hyphæ similarly isolated, and, at the particular stage desired, the cultures were treated as described above. This process allowed of the fixing and staining of specific hyphæ whose development had been followed.

III. *Observations on Living Material.*

A. Hyphal-Fusion.—The mycelia produced from sporidia isolated from segments of a promycelium, grow mainly on the surface of the nutrient agar and consist of secondary sporidia (or conidia) and hyphæ, the proportions of which vary according to the medium on which they are growing. The conidia increase by budding, or may give rise to hyphæ. The hyphæ grow at their apices, cutting off behind them cells which may or may not have contents. The cells with contents may produce conidia or new hyphæ.

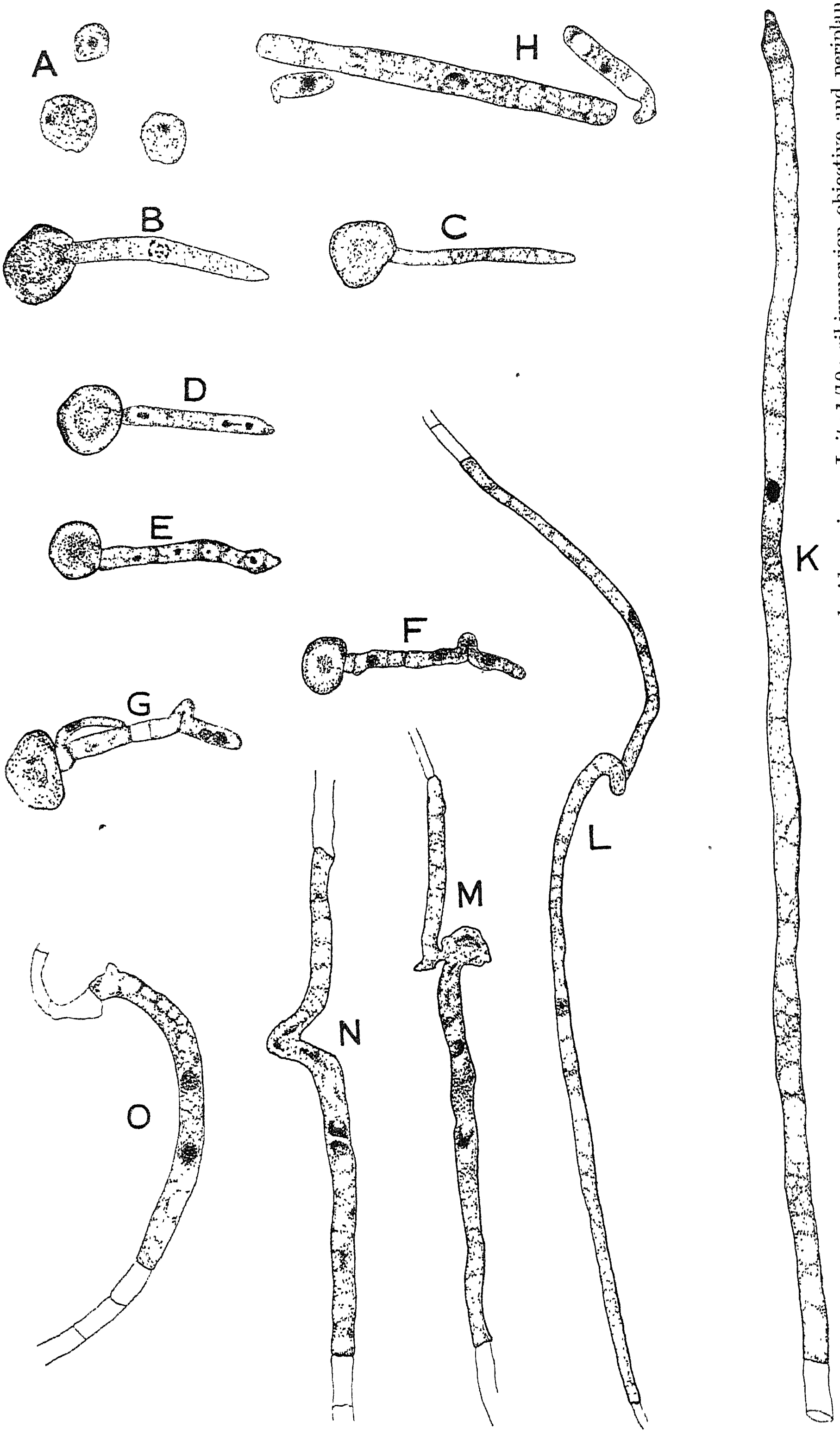
When two mycelia derived, each from a single sporidium, were allowed to meet on beef extract agar, one or other of two appearances was seen. Either the hyphæ of one mycelium grew past the hyphæ of the other, quite irrespective of their presence (Plate 10, Photo. 5), or, the hyphæ of the two mycelia, after approaching within a certain, but rather indefinite distance of one another, curved towards each other in a clear and definite manner (Plate 10, Photo. 3), suggestive of chemotactic stimulation. In Plate 10, Photos. 1–4, a meeting of hyphæ of the latter type is shown, the photographs being taken of the same hyphæ at intervals of one hour. The curving is not characteristic of the hyphæ of one of the mycelia only, but may take place in the hyphæ of both. It has been seen only at the meeting of two mycelia.

In this second case the hyphæ, on coming into contact, swell, become more translucent, often taking on a slight brown tint, and, as tested with the needle of the Isolator, are stiffer than ordinary hyphæ. In certain instances the contents of both hyphæ after contact suddenly disappeared, often in less than a second, leaving only the cell walls. In most cases that have been observed, however, the contents pass completely from one hypha into the other, leaving only the empty cell on one side of the point of union. The result is a cell containing the protoplasmic content of two cells of different origin, and to this the term fusion-hypha has been applied.

This fusion would appear to be homologous with that occurring between the germ tubes of sporidia, and in the clamp connections in the promycelium described in previous work on the Smuts ; and would appear to come under Winkler's category of " Pseudomixis " (Sharp 6). Kniep (1) used the letters " A " and " B " to distinguish his " sexes," and in this work the same practice is followed. As, however, the term " sex " has been used by different authors with different connotations, and as, in the present organism, the hyphæ which fuse show no morphological differentiation as gametes, the non-committal term " gender " has been adopted in lieu of the term " sex."

Isolations of the sporidia from 82 promycelia have been made, and with the exception of three isolations, which were made before the technique had been improved to its present standard, it was found that two of the sporidia of each promycelium were of one gender " A " and two were of another gender " B." In a subsequent paper it is proposed to consider the results of these isolations with an analysis of their gender reactions.

B. Growth after Fusion.—When the fusion-hyphæ were kept under observation for one to three or more days, they were found either to remain inactive or to grow out as hyphæ. In an old culture the fusion-hyphæ always remained



Text-fig. 1.—All drawings in this figure have been made with the aid of a camera lucida, using a Leitz 1/10*a* oil-immersion objective and periplan : oculars 6, 10, 12. Cells drawn in outline are those with no contents.

A.—Chlamydospores of Covered Smut of Oats with the spore coat removed, showing uni-nucleate condition. × 890.

B–E.—Stages in the germination of the chlamydospore with the formation of a promycelium, and the divisions resulting in four nuclei. × 890.

F–G.—Clamp connections being formed between the uni-nucleate segments of the promycelium. × 890.

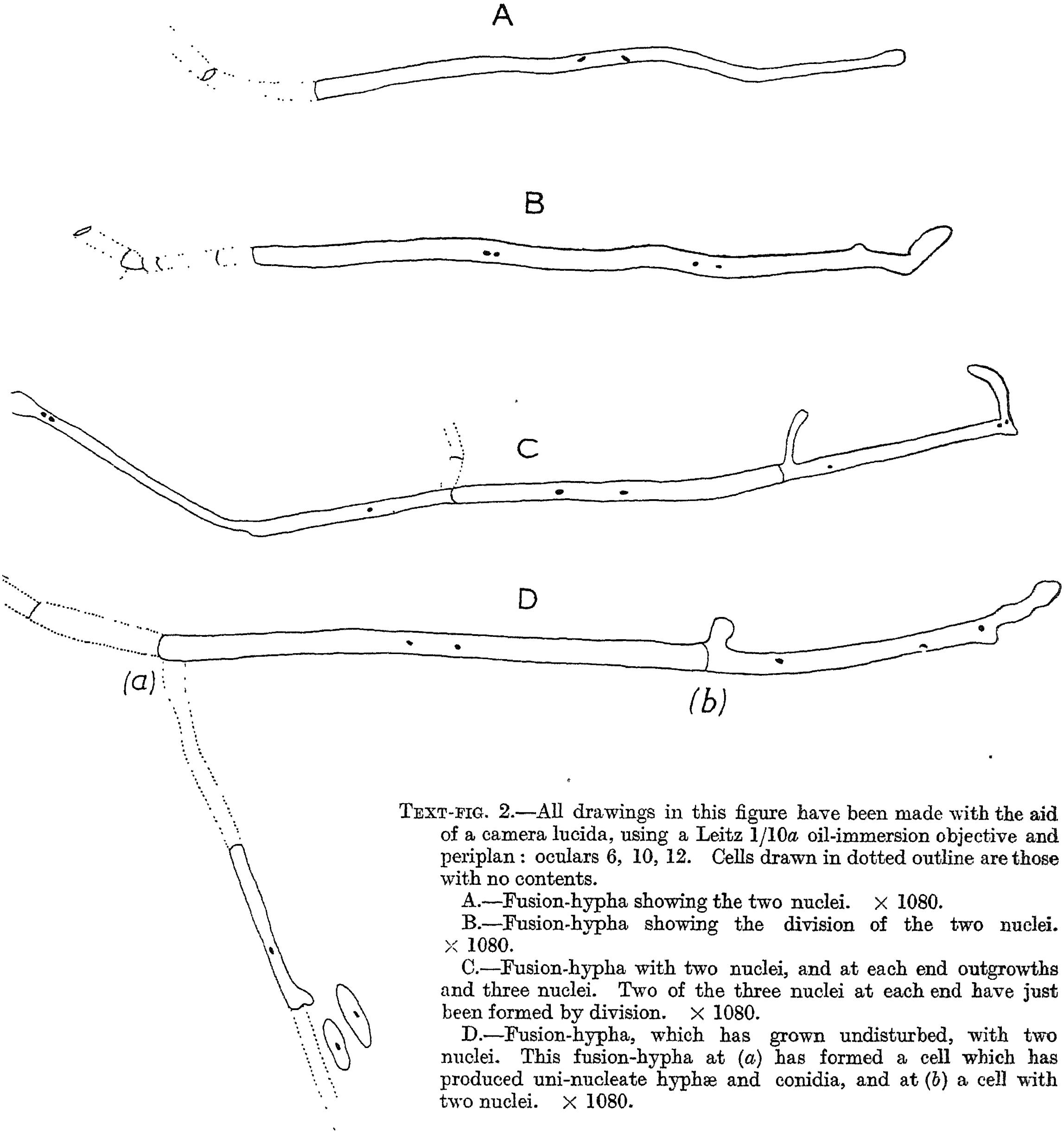
H–K.—Uni-nucleate hyphæ and conidia, characteristic of the mycelia arising from single sporidial isolations. × 1340.

L.—Two hyphæ of different gender in contact with each other, showing the characteristic curving. × 890.

M–N.—Two hyphæ, after fusion, showing both the nuclei in one of the hyphæ. × 890.

O.—Fusion hypha showing the contents of both the original hyphæ on one side of the point of fusion. × 890.

inactive, but if they were moved by the Isolator to a fresh part of the agar, or if, on the other hand, they had been formed in a young culture, they proceeded to grow in one of two ways. Sometimes they continued to grow, branching occasionally and resembling in every way the original fusion-hypha, and leaving



Text-fig. 2.—All drawings in this figure have been made with the aid of a camera lucida, using a Leitz 1/10*a* oil-immersion objective and periplan: oculars 6, 10, 12. Cells drawn in dotted outline are those with no contents.

A.—Fusion-hypha showing the two nuclei. × 1080.

B.—Fusion-hypha showing the division of the two nuclei. × 1080.

C.—Fusion-hypha with two nuclei, and at each end outgrowths and three nuclei. Two of the three nuclei at each end have just been formed by division. × 1080.

D.—Fusion-hypha, which has grown undisturbed, with two nuclei. This fusion-hypha at (*a*) has formed a cell which has produced uni-nucleate hyphæ and conidia, and at (*b*) a cell with two nuclei. × 1080.

behind them empty cells and, from time to time, cells with contents. After several days, in all such specimens observed, they either died or stopped growing. In other cases conidia and hyphæ were formed at one or at both ends of the isolated fusion-hypha, and these eventually produced a new mycelium. When conidia or hyphæ were isolated from one end of the fusion-hypha they were found to be of the same gender, but when they were isolated from opposite ends of the fusion-hypha they were of different gender. These isolations have been repeated a number of times. It was also noted that fusion sometimes occurred between the conidia and hyphæ arising from different ends of the same fusion-hypha, and also that a hypha from one end of the fusion-hypha could sometimes be observed conjugating with the other end of the fusion-hypha itself.

In the earlier part of this work fusion-hyphæ were isolated and transferred direct to media in test tubes and, after sub-culturing, their gender reactions were tested. It was found that except in two isolations each culture was wholly of one gender. In the first exception, No. 5, no differentiation was visible in the macroscopic appearance of the culture, but the reaction was both "A" and "B," according as different individual hyphæ of the one mycelium were tested. Single sporidial isolations from this culture gave a definite gender reaction, either "A" or "B," but not both. In the second exception, No. 39, the upper part of the mycelium in the test tube was white and dry, composed largely of hyphæ and was of "A" gender; the lower part was brown and slimy, largely sporidial and was of "B" gender.

These observations imply that the culture arising from an isolated fusion hypha is usually of one gender, and only occasionally are both genders found in such cultures.* Both genders, however, can be obtained from the conidia and hyphæ growing out from an isolated fusion hypha, and hence it must be concluded that the survival of one gender in a culture is due either to a differential growth rate or to inactivation of one of the genders.

IV. *Observations on Fixed and Stained Material.*

(A) *Hyphal-Fusion.*—A study of the cytology shows that the behaviour of the nuclei is consistent with the phenomena described above. A developing mycelium consists of uni-nucleate hyphæ growing at their apices and cutting off cells, usually empty, behind them. (Text-fig. 1, H.K.) At times one of these cells may contain a nucleus and cytoplasm, and this cell may then give rise to conidia or to a fresh hypha. When hyphæ of different gender come into contact they either fuse immediately or twine or coil once or twice and then fuse at their tips (text-fig. 1, L.). As soon as the portion of hyphal wall separating the cytoplasms has disappeared, the contents pass from one hypha into the other and the two nuclei approach one another and lie side by side (text fig. 1, M.N.O.). In the meantime the cell wall of the fusion hypha becomes slightly thicker, the hypha increases in diameter and its cytoplasm retains hæmatoxylin stain longer than that of the ordinary hypha. Such a hypha, which may be called a "fusion-hypha," has only been observed as a result of the process of conjugation.

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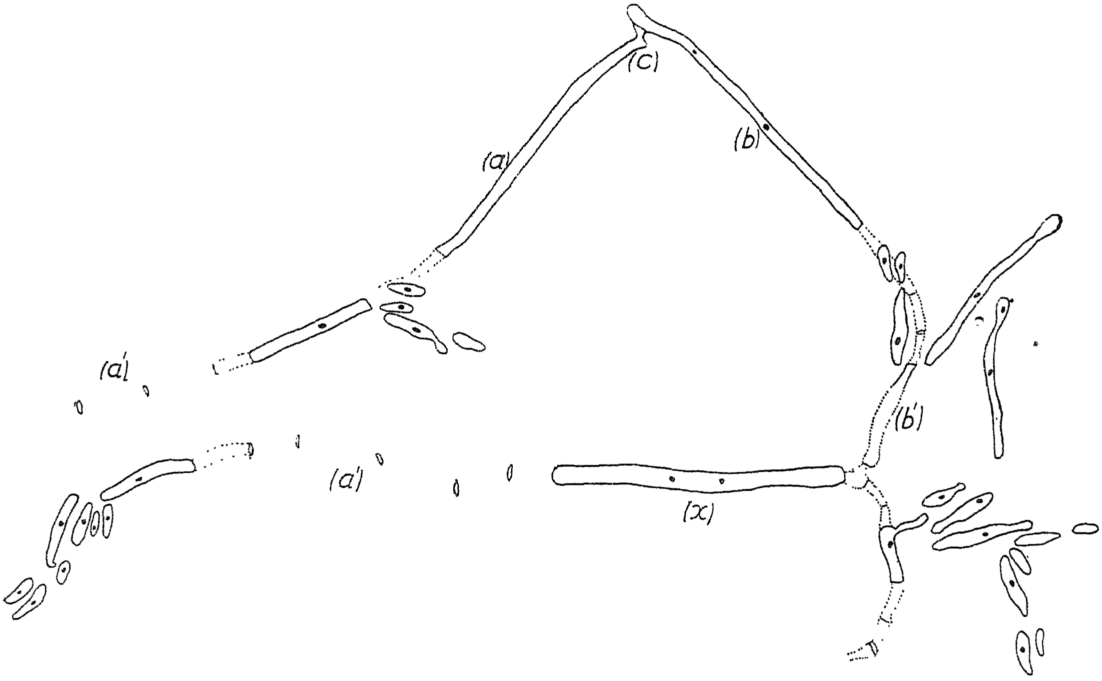
* As in the earlier isolations, the gender reactions were only tested after sub-culturing, it is possible that in some cases one of the genders was lost in this process.

This type of fusion of hyphæ has been observed in the covered smuts of oats and barley, not only between the hyphæ of each species, but also between the hyphæ of one of these species and the hyphæ of the other species; and every observation of this type of conjugation, where two hyphæ only are concerned, has shown the immediate fusion-hypha with two nuclei. Nothing has been seen in the preparations which suggests the fusion of nuclei in a fusion-hypha.



TEXT-FIG. 3.—Drawing made with the aid of a camera lucida, using a Leitz 1/10*a* oil-immersion objective and periplan: ocular 10. Cells drawn in dotted outline are those with no contents.

The original fusion-hypha is shown at *x*. At each end it has given rise to uni-nucleate hyphæ and conidia. The connection between the hypha (*a*) and the fusion-hypha is shown by the transverse walls of the empty cells (*a′*). The connection between the hypha (*b*) is shown by the dotted outline of the empty cells (*b′*). The two hyphæ (*a*) and (*b*), one from each end of the fusion-hypha, have fused at (*c*), and the nucleus from (*a*) has moved into the hypha (*b*). × 1080.

In certain specimens three or more hyphæ have apparently fused; and in one example of a triple fusion, where the fusion-hypha could be stained, three nuclei were present. This would appear to be the same phenomenon which Kniep (3) has described in living material.

(B) *Growth after Fusion.*—The cells of all the mycelia arising from isolated fusion-hyphæ were uni-nucleate, except that of No. 5, where, in addition, cells, similar to fusion-hyphæ with two and more nuclei, were also present.

Isolated fusion-hyphæ were grown, their development watched, and at the appropriate time they were fixed and stained. The fusion-hypha has been found to be at first bi-nucleate (text-fig. 2, A). Each of these two nuclei of the fusion-hypha divide (text-fig. 2, B), and two of the products of this double division remain in the centre, while one passes to each end of the hypha; a wall is then formed on either side of the two central nuclei. The single nuclei in the new cells at either end divide frequently and uni-nucleate conidia and hypha grow out. In text-fig. 2, C, the nuclei at each end have divided once, and one of these at each end has divided again. In the meantime outgrowths at the ends of the fusion-hypha have been formed. In text-fig. 2, D, there is shown a fusion-hypha which has not been moved with the Isolator but which has grown completely undisturbed. This fusion-hypha at (*a*) has cut off a uni-nucleate cell which has grown out into a hypha, while at (*b*) it has formed a hypha with two nuclei.

A number of experiments were made on materials where hyphæ had been formed at each end of the fusion-hypha and were still attached by the walls of the empty cells. Such experiments consisted in moving, by means of the Isolator, the hyphæ until their further growth would bring them into close proximity. A typical example of the result is shown in text-fig. 3. Here the two hyphæ so brought together have fused at " C," and the nucleus of one has passed into the other hypha. The connections of these hyphæ with the original fusion-hypha are still shown by the transverse and longitudinal walls of the empty cells.* This stage, showing fusion of hyphæ or conidia from the two ends of a single fusion-hypha, has been fixed and stained in a number of specimens, and it is on the observation of these that fusion has been determined.

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* In the present condition of this slide, the cross walls only can be seen in the case of one of the hyphæ (*a*′). The reason for this is that the amount of de-staining which is necessary to differentiate the nuclei removes the stain almost entirely from the walls of the empty cells.

V. *Conclusions.*

The conclusions drawn from these observations are as follow :—The fusion of two hyphæ of different gender results in a cell containing two associated nuclei, one of " A " gender and the other of " B " gender. This bi-nucleate cell produces a hypha, under certain conditions, into which the two nuclei pass. After some growth of this hypha the two nuclei divide, and, of the four products of division, one nucleus of " A " gender passes to one end of the hypha, two nuclei. one of " A " gender and one of " B " gender, remain associated in the centre, and the fourth nucleus, of " B " gender, passes to the other end of the hypha. In this way an order A, AB, B, or *vice versa*, is obtained in the hypha. The cells containing single nuclei produce uni-nucleate conidia and hyphæ. Since the single nuclei are of different gender, a new fusion-hypha results when two of the hyphæ from opposite ends of the fusion-hypha are brought together.

I wish to thank Dr. W. B. Brierley for much helpful criticism and encouragement during the course of this work.

Summary.

The cytology of the covered smuts of oats and barley in pure culture has been investigated, and the fusion, both within and across the species investigated, between the mycelia of different " gender " derived from single sporidial isolations is described.

The fusion-hypha is bi-nucleate, and nothing has been seen which suggests that nuclear fusion occurs. The bi-nucleate fusion-hypha gives rise to uni-nucleate hyphæ which are of different gender, these being produced at different ends of the fusion-hypha.

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DESCRIPTION OF PLATE 10.

All photographs have been taken with a Leitz "Micca" camera, with a No. 6A Leitz objective and a periplan : ocular 10, and enlarged.

PHOTOGRAPHS 1–4.—These are a series of photographs taken at intervals of one hour, showing the meeting of the hyphæ of two mycelia of different gender. The curving (see hyphæ within circle) which is most marked in Photograph 3, is characteristic of such a meeting.

PHOTOGRAPH 5.—This is a photograph of the meeting of hyphæ of two mycelia of the same gender.

The History of the Cytoplasmic Inclusions of the Egg of Ciona Intestinalis (L.) *during Oogenesis and Fertilisation.*

By L. A. HARVEY, M.Sc., A.R.C.S., Assistant in the Department of Natural History and Zoology, University of Edinburgh.

(Communicated by Prof. E. W. MacBride, F.R.S.—Received October 16, 1926.)

[PLATES 11–13.]

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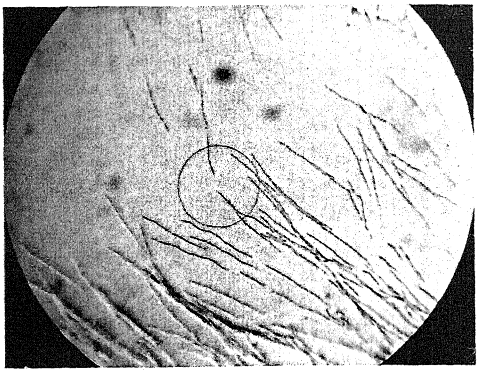
§ 1. *Introduction.*

In a previous paper the process of yolk formation in the oogenesis of *Lumbricus terrestris* has been investigated (10). In the present paper the history of yolk formation and of the cytological constituents of the oocyte of *Ciona intestinalis* is traced, and an attempt is made to correlate the functions of these bodies with the metabolism of the cell.



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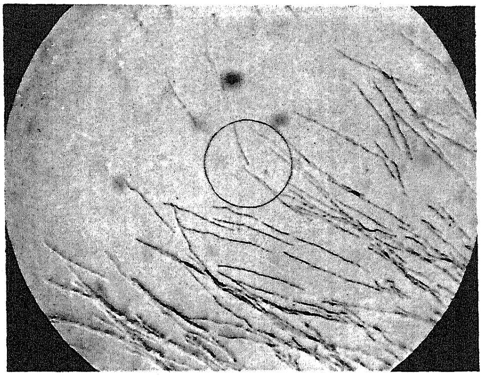

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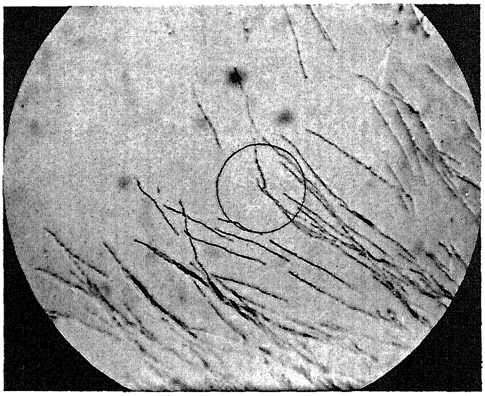

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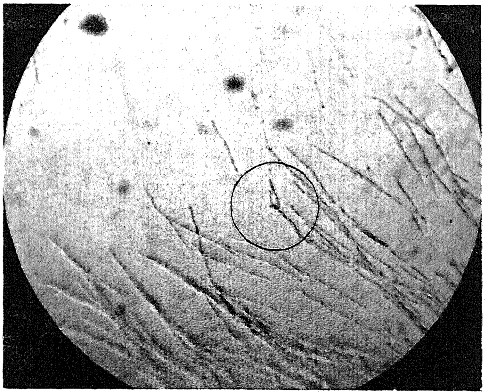

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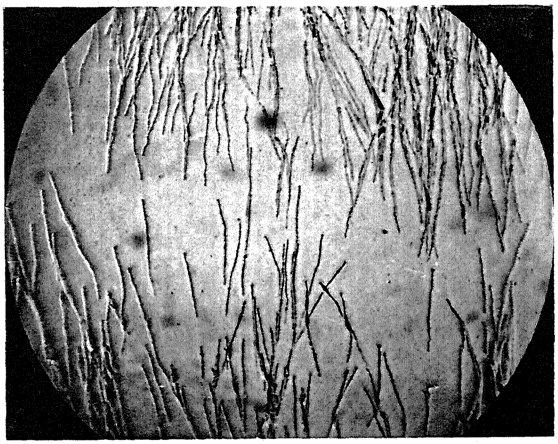

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London Stereoscopic Co. Imp.

It had originally been intended to follow the inclusions from the early oocyte up to the cells of the young Ciona immediately after the metamorphosis of the larva. For this purpose material, both ovarian and larval was collected. But on examination of the embryological material it was found that little of essential importance could be added to the results published by Duesberg (8, 9) for the yolk and mitochondria, and almost complete failure was met with in all attempts to impregnate the Golgi apparatus in the larval cells. In consequence attention has been concentrated on the ovarian material and upon the changes occurring in the egg after it has left the ovary, up to the time of fertilisation and of the first cleavage.

The question of yolk formation is of paramount interest in the study of oogenesis. It is one of the problems in cytology the solution of which will lead to a clarification of ideas on the relative functions of the various components of the cell. Bowen (2) recently has suggested that yolk formation may be the chief function of the Golgi apparatus of the egg, corresponding to the formation of the acrosome in the sperm.

The Golgi apparatus has been recognised by many authors as playing a part in the formation of certain kinds of yolk in the egg, the fatty yolk in many Arthropoda and Mollusca, etc. (see *infra*). But as a rule the major part of the true yolk in the egg seems to be formed by some means in which the Golgi apparatus plays little part. Usually either the mitochondria or the nucleolar extrusions have been cited as forming the primordia from which the yolk droplets develop. It is hoped, however, that the present work will show an intimate relation between the yolk of Ciona and the Golgi apparatus.

It is a great pleasure to the author to be able to acknowledge his indebtedness to Prof. E. W. MacBride, F.R.S., of the Imperial College of Science, London, where this work was commenced, and also to Prof. J. H. Ashworth, F.R.S., of the University of Edinburgh, where it was finished, for the help and advice which they so freely accorded him. To the Committee of the Beit Scientific Research Fellowships for the award of a Fellowship, and to that of the Moray Fund in the University of Edinburgh for a grant in aid of expenses, the author wishes also to express his indebtedness.

§ 2. *Literature.*

Recent work on yolk formation points to the existence of two main types of yolk in the egg: true, non-fatty yolk and fatty yolk. These two types may be present together in the eggs of many Arthropoda and Mollusca, where the phenomena of oogenesis have been most intensively studied. The evidence points to the origin of the fatty yolk directly from the Golgi elements and of the true yolk either from the mitochondria or from the nucleolar extrusions. Evidence on yolk formation in other groups has been derived from the study usually of but one form, and the results at the present time are very contradictory. Yolk is stated to be formed from mitochondria, Golgi apparatus, nucleolar extrusions, under the influence of, but not directly from, the mitochondria or Golgi apparatus, or both, from the cytoplasm.

This state of confusion is very unsatisfactory, and, while some cytologists, notably Wilson (20), lean towards the view that yolk includes many substances which are deposited in the egg during its growth, arising from many different sources, others, including Bowen (2), suggest that there must be some uniform method of yolk formation in eggs, and that many observations recorded so far must be either faulty or lacking in that detail necessary for final correct interpretation.

A summary of the literature on yolk formation will be reserved for a later date when further observations have been made which it is hoped will throw light on the general problem. Only those papers dealing with Ascidian oogenesis will be dealt with here.

The most comprehensive study of the oogenesis of an Ascidian is Hirschler's paper (11) on *Ciona intestinalis*. He found that yolk droplets are formed by the swelling of the mitochondria : later they receive lipoid material by deposition from the Golgi apparatus, whose elements, as they are dispersed in the cytoplasm from a localised area, fuse with the yolk droplets. The yolk nucleus is of cytoplasmic origin. It gives rise to the mitochondria and then undergoes complicated changes, acquires a connection with the nuclear chromatin, and finally disappears in the cytoplasm. This work will be criticised under the various following sections, where more detailed summaries will be given.

Crampton (7) concludes that the yolk nucleus of *Molgula manhattensis* is of nuclear origin, from the albuminous nature of both. Yolk arises by the swelling of the yolk nuclear granules, after their dispersal in the cytoplasm. The relation between the nucleus and yolk formation is also claimed by Schaxel (17), who describes the emission of chromatin from the nucleus, converting the primary achromasy of the cytoplasm into chromasy. Yolk is formed in the chromatic bodies so produced. The remainder of the chromatin forms a superficial mantle inside the test-cells.

Loyez (13), as quoted by Cowdry (5), has described the process of yolk formation in Ciona, apparently in some detail. In the young oocyte the mitochondria are few and granular, lying round the germinal vesicle and peripherally. They increase in number and are distributed in linear series in strings in the cytoplasm. They swell and yolk is deposited in them. A certain amount of fusion takes place and the final product is yolk. This process is not found in *Cynthia tetraëdra* or *C. morus*.

According to Bluntschli (1) yolk formation commences centrally among the mitochondrial threads formed by the dispersal of the acidophil cytosomes, reaches a climax, and ceases. It is followed by a second peripheral yolk formation, when yolk droplets appear and grow among the fused mitochondria. The mitochondria multiply in the yolk-free zone round the nucleus, and lie in between the yolk spheres. His observation of peripheral yolk formation is confirmed by Wernicke (19) who states that the peripheral basi-chromatic bodies left after central yolk formation absorb nourishment from the test cells and become yolk.

Recently Parat and Bhattacharya (16) have published a note on observations of the vital staining of the oocyte of Ciona with neutral red, etc. They claim to have confirmed Hirschler's description of the mitochondria and Golgi apparatus, and also of the origin of yolk from mitochondria and Golgi elements. The Golgi apparatus is represented by the vacuome, consisting of vacuoles whose contents have an acid reaction. The yolk nucleus was not found, and, in consequence of observations which are not recorded, it is considered to be an artefact.

There are few observations on the changes in the egg after breakdown of the germinal vesicle and before the union of the male and female pronuclei. The earliest are those of Conklin (3, 4). They are recorded from the point of view of the localisation of " organ-forming substances " in the egg. The work was carried out mainly on *Cynthia partita*, but was confirmed on *Ciona intestinalis*. In the ovarian egg there is a peripheral layer of protoplasm free from yolk and full of pigment granules (Cynthia). The test-cells lie here. Centrally lies yolk-laden protoplasm with an excentric, large, clear germinal vesicle. The chorion is formed and the test-cells are extruded to lie between it and the surface of the egg. When the egg leaves the ovary the germinal vesicle bursts and the nucleus enters on the early phases of maturation, reaching the first metaphase, at which point it stops until the entry of the sperm. This enters at the vegetal pole, and immediately the peripheral protoplasm flows down and forms a cap round it. The clear substance of the germinal vesicle also flows down and forms a cap beneath the cap of peripheral protoplasm. At the opposite pole is collected the yolk, and here the polar bodies are thrown off. The sperm nucleus and aster move up the posterior side of the egg taking the caps of protoplasm, and these finally form two crescents lying symmetrically below the equator, in a plane at right angles to the future long axis of the larva. Subsequently the materials of the two crescents and the various regions of the egg are distributed with precision to the organs of the larva.

These results have been analysed by Duesberg (8, 9) on *Ciona intestinalis*, and by Meves (14) on *Phallusia mammillata.* Both workers agree in denying the presence of the clear area of nucleoplasm in the egg, and describe the crescent of peripheral cytoplasm as composed of mitochondria. Meves describes the mitochondria, both those of the crescent and those lying among the yolk droplets, as vesicular. The upper and lower surfaces of the crescent are deeply indented, the indentations being filled, on the upper surface with yolk droplets, on the lower with hyaline protoplasm.

Duesberg confirms Meves' description of the empty protoplasm outside the mitochondria of the crescent. He finds no difference in zoning between the fertilised and unfertilised egg, but beneath the crescent describes the yolk as being less densely packed, the droplets being arranged in rows as are also the mitochondria. As regards the nucleus he finds that it may still be in early prophase at the time of insemination. He confirms Conklin's account of the lack of both centrosome and astral rays, and also as to the movements of the sperm and egg nuclei prior to the formation of the primary karyokinetic spindle. The allotment of the various regions of the egg to the specific organs of the larva is also confirmed.

§ 3. *Material and Methods.*

The material used in this work was collected partly in the Marine Biological Laboratory at Plymouth, and partly in London and Edinburgh from living animals sent from Plymouth. The author would like to take the opportunity to record his appreciation of the service rendered him by the director and staff of the Plymouth Laboratory, both while he was there and also in sending living animals to London and Edinburgh.

For the study of the oogenesis the test was slit longitudinally, the animal squeezed gently out, and entirely removed from the test. The ovary exposed in the fold of the gut was quickly removed, cut into three or four pieces, and transferred into the following solutions: for mitochondria to Champy's fluid, to Kolatschev's modification thereof, to Flemming without acetic acid, to Nassonov, etc. The Kolatschev material was treated, some with osmic acid, as was originally intended for the method, some with pyroligneous acid mixture as for Champy. For the Golgi apparatus reliance was placed upon Da Fano's cobalt nitrate-formol method. Osmic methods were used as a check on these results, but, as has been stated in a previous paper (10), these techniques make the tissue liable to shrink and become brittle, and in addition give a less extensive picture of the apparatus than do the silver methods. Also the presence of a large amount of lipoid in the ovary, localised largely in the fully grown oocytes packed with yolk, renders the osmic methods capricious, good impregnations being the exception rather than the rule, and the amount of non-specific reduction is relatively great.

For general fixation, cytoplasmic staining affinities, and nuclear details, material fixed in Bouin's picro-formol was used. Mitochondrial material was stained in iron hæmatoxylin or anilin fuchsin followed by methyl green, or by aurantia and toluidin blue. Ehrlich's hæmatoxylin was used as a counterstain after the silver technique and as a general stain after Bouin.

For embryological stages ripe eggs were removed from the oviducts and fertilised in seawater with sperm from other individuals. Stages were fixed in the above-mentioned fluids at the following intervals after insemination—1, 2, 5, 10, 20, 30 minutes. After that batches were fixed at the 2-, 4-, 8-, 16-, 32-cell stages, etc., up to the metamorphosing larvæ. Unfertilised eggs were also fixed direct from the oviduct and after 10 to 30 minutes in sterile seawater.

Unfortunately, as has been stated, attempts to demonstrate the Golgi apparatus in the developmental stages of the larva have met with an almost complete failure. A few specimens have been found here and there in which a certain amount of impregnation has taken place, but these have given an imperfect series which has sufficed to show that the distribution of this organ in the cells of the larva is quite regular. Both silver and osmium methods were equally unsatisfactory. The various stages of this series were embedded in celloidin made from a mixture of clove oil and alcohol-ether solutions. The blocks so obtained were trimmed and embedded in paraffin, of melting point 56° C., and cut at 3 to 5 μ.

§ 4. *The Mitochondria.*

In his account of the oogenesis of *Ciona intestinalis* Hirschler (11) derived the mitochondria of the oocyte from the same source as the yolk nucleus. In the very young oocyte there are no individual mitochondria to be found, but after a certain period acidophil granules appear, apparently passing out from the surface of the yolk nuclear body, which is losing its intensity of staining owing to the emission of these mitochondrial granules. From the neighbourhood of the yolk nucleus they gradually spread over the entire cytoplasm, multiplying in numbers until the cell is full of them except in the area occupied by the nucleus, Golgi apparatus and yolk nucleus. The latter then migrates peripherally where it fragments and disappears. The yolk nucleus is present in the earliest oocyte as a very small acidophil granule lying close to the nuclear membrane. It enlarges and soon acquires a capsular form owing to the decrease in staining power of the interior as the mitochondria pass out. Sometimes there are several layers in the wall. The capsule may be completely closed, or it may be open at one side, but there are never mitochondria inside it. A stalk grows from it to the nuclear membrane and a connection is established with the nuclear chromatin, whereby the latter passes into the yolk nucleus. Finally the stalk disappears and the yolk nucleus itself breaks up and is dissolved in the cytoplasm. After they have become distributed in the cytoplasm, all the mitochondria, except a few scattered ones and those forming a narrow peripheral layer, swell up and form yolk droplets, which stain with the same dyes as the primordia from which they originated. The Golgi apparatus now fragments and becomes scattered in the cell. As it spreads so the yolk droplets are blackened, after osmication, owing to deposition of lipoids in them by the Golgi elements which become applied to their surfaces.

Hence the yolk, according to this author, is derived from the mitochondria, which become extremely distended, and then a secondary deposition of lipoid in the droplets takes place and completes its formation, involving both the mitochondria and the Golgi apparatus. The test cells, which are full of lipoidiferous granules, take no part in the process but remain unchanged.

There are three points in this description which it was thought would repay investigation of the problem. Firstly, the origin of mitochondria and yolk nucleus from a common body in the cell is unusual; secondly, the function of the test-cells, if not nutritive, requires elucidation, for they are thrown out of the cell when it is ready for fertilisation, and lie between the egg and the follicle as an apparently idle layer; lastly, the question of the part which the yolk nucleus plays in yolk formation, and of why should it pass through the very complicated evolution described by Hirschler and then simply fragment and disappear. In the present investigation of the oogenesis particular attention has been paid to these points, and the conclusions drawn, as to the relative functions of the bodies present in the cell, differ radically from those at which Hirschler arrived.

In the first place the mitochondria were found to be present in the earliest oocyte as bodies having no connection whatever with the yolk nucleus. The very early oocyte, in the germinal epithelium (Plate 11, figs 1, 2, 3), has a slightly oxyphil cytoplasm somewhat flocculent in consistency even after the best obtainable fixation. This renders detection of the mitochondria extremely difficult, but in most cases they can be seen as a clump of not more than six to eight vesicles with a chromophobe interior bounded by an aura of acidophil material which seems to have no definite outer limit, but fades into the ground cytoplasm. These are lying usually about midway between the nuclear membrane and the periphery of the cell, on the side of the excentric nucleus to which most of the cytoplasm is aggregated. Hence, as is probably always the case, the mitochondria are present in the oocyte from its beginning, being passed on from the oogonium, and do not arise *de novo* in the cytoplasm. Nor are they in any way associated with the yolk nucleus, the history of which will be described later.

After the stage of primary oxyphily of the cytoplasm it enters upon a period of strong basophily, and at the same time becomes much denser and less flocculent in consistency. During this period, after anilin fuchsin and methyl green, the cytoplasm stains a dense slaty blue colour, owing to the absorption of a certain amount of fuchsin as well as a large amount of methyl green. Against this ground colour the mitochondria can be seen with rather more ease than before, but owing to their extreme delicacy their identification is still by no means an easy task. Their number has increased to about sixteen or twenty when the cytoplasm becomes definitely basophil. As the cell enlarges, which it does usually by elongation along one axis, the mitochondria increase in number and tend to spread in a zone around the nucleus (Plate 11, fig. 4).

No figures that might be interpreted as divisions of the mitochondrial granules have been found, but from the fact that fresh mitochondria always arise near their fellows it may be safely concluded that they are derived from these latter, and not from the cytoplasm or any other element in the cell.

The period of basophily does not last long, but is early followed by a secondary period of oxyphily in which the cytoplasm stains uniformly with acid dyes and exhibits little if any structure at all. The mitochondria now form an irregular hollow sphere surrounding the nucleus, Golgi apparatus and yolk nucleus (Plate 11, fig. 5). The mitochondrial spherules stand out clearly owing to the greater intensity with which the acidophil auræ now stain.

The yolk nuclear bodies lie among the mitochondria, but never is there a sign of the investing palisade of mitochondria on which Hirschler has based his description of the common origin of the two elements. The association of the two elements seems to be entirely fortuitous, comparable to that found in the pallial layer of many other animal eggs.

The mitochondria give the impression of lying at haphazard on an invisible reticulum. They are frequently arranged in short rows, or are clumped together as if on the nodes of a network. Frequently strands of a darkly staining material can be seen underlying the mitochondria, joining two clumps together and passing out towards the periphery of the egg as if pre-determining the lines along which the mitochondria shall be formed. In Kolatschev material, which has been osmicated, the cytoplasm around the mitochondria contains an extraordinary deposit of fine blackened granules and threads which apparently correspond to the semi-invisible reticulum just described. The complex then has the appearance of a spider's web hung from points in the periphery of the cell about the clear nucleus. This appearance has not been obtained with any other mode of preparation of the cell.

Soon the cytoplasm becomes evenly strewn with mitochondria, with perhaps a denser accumulation towards the periphery (Plate 11, figs. 5, 6, 7). The yolk nuclear material begins to fragment and disappear, and at the same time changes are seen in the mitochondria. The acidophil aura of each element begins to disappear, and finally the element consists of a chromophobe vesicle surrounded by a sharply defined acidophil rim. This suggests that there is a change in the surface relations between the vesicle and its external medium, whereby the acidophil material of the aura is adsorbed on to its surface to form a definite rind. Possibly there is an increase in the lipoid content of the vesicles, which affects the adsorption phenomena in these latter. This change is suggestive in view of further events which now take place. The vesicles increase in size somewhat, and then a differentiation into two categories commences. The first category consists of mitochondria which remain exactly as they are. These lie sparsely scattered over the entire cytoplasm, but are densely packed, to the exclusion of all other elements, at the periphery, between and around the test-cells. The mitochondria of the second category enlarge and become uniformly stained, the intensity of staining varying among individual granules, but tending to become weaker after a certain size has been reached during the process of enlargement. The spheres so formed are the yolk droplets, and they lie closely investing the nucleus and filling the egg except, as before mentioned, the peripheral layer (Plate 11, fig. 8).

This process of yolk formation may commence round an area to one side of the nucleus, or become visible as a change in the mitochondria all over the cell, or at one end of the cell from which it spreads to the other. Always from its point of origin it passes progressively throughout the cell until the whole of the yolk is formed.

The cell, after completion of this process, consists of a large germinal vesicle containing a large nucleolus, surrounded by a broad area containing many closely packed yolk spheres of varying size and staining capacity. Among these spheres lie a number of vesicular mitochondria as chromophil-rimmed spheres. Surrounding the yolk is a thin zone of cytoplasm packed with vesicular mitochondria, and here lie the nests of test-cells which are full of vacuoles left by the disappearance of the osmiophil granules which packed them before the commencement of yolk formation. A layer of vacuolated follicle cells invests the whole in a complete mantle.

The final change in the mitochondria follows the nuclear changes preparatory to maturation. The nuclear membrane breaks down; the nucleolus fragments and the germinal vesicle disappears (Plate 12, fig. 9). Soon after this the test-cells are extruded from the egg, which shrinks in size somewhat. This is accompanied by a slight concentration of the mitochondria towards the vegetal pole. Before this change the mitochondria form a complete, irregular, peripheral layer lying just internal to the test cells. When the test cells are extruded the layer becomes discontinuous over the animal pole, and here the yolk reaches to the surface of the egg. At the vegetal pole there is now a sharply defined layer of mitochondria which extends as a gradually narrowing rim up to and beyond the equator, covering about three-quarters of the surface of the egg.

During the change recorded above the mitochondria shrink somewhat and lose their colourless interiors, becoming small, faintly-staining granules. The peripheral layer of mitochondrial vesicles thus becomes a crescent or rather a cup of granules, and at the same time the egg appears much denser than before these changes. This is probably due in part to the lack of light relief, formerly provided by the germinal vesicle, but also to a certain extent to the deeper staining capacity of the yolk.

Before the egg leaves the ovary the reconstructed nucleus usually reaches the stage of the formation of the metaphase spindle in preparation for the first maturation division. At this stage the spindle is fairly closely invested with yolk, but it lies in a narrow clear zone, where there is an accumulation of mitochondria as two hollow irregular spheres, one about each pole of the spindle, as if orientated about a central body of some sort, but no central body can actually be seen (Plate 12, fig. 13).

§ 5. *The Golgi Apparatus.*

Hirschler (11) has followed the history of the Golgi apparatus in osmic acid preserved material. He found that there are three distinct phases passed through by this material during oogenesis, corresponding to three physiological phases passed through by the cytoplasm as indicated by changes in its staining affinities. In the very young oocyte he found a few small osmiophil bodies lying on the nuclear membrane. These are in the form of rings or half-rings, probably the optical sections of spherules, etc. They are dotted at intervals round the nucleus and there is no concentration about any particular area. This he called the primary diffuse stage. As the oocyte grows the elements concentrate to one side of the nucleus and commence to come close together. Fine osmiophil threads connect the elements into a network or lamellar platework, which usually lies close to the yolk nucleus at this period. This he called the complex stage.

After this the secondary diffuse stage is reached by the gradual fragmentation of the network into discrete elements, and the dispersal of these among the yolk droplets, now forming. As these Golgi elements pass away from the area formerly occupied by the complex apparatus they become applied to the surfaces of the yolk spherules and deposit lipoid material there. Thus while the more peripheral yolk spheres stain red with acid fuchsin, there is an ever-widening zone of blackened yolk which finally absorbs the red zone completely, as the Golgi apparatus reaches its limit of dispersal.

In the present investigation it has been found that Da Fano's silver impregnation method, while not giving so complete a picture of the cell, gives a more complete idea of the Golgi apparatus than do osmic methods. By correlation of the results obtained by silver and mitochondrial methods the relations of the Golgi apparatus to other cell inclusions can be discovered.

In the main the results obtained by silver methods confirm Hirschler's findings, but the silver reveals the presence of a greater amount of Golgi apparatus material than does the osmic. In the young oocyte immediately following the oogonial stages there are, as Hirschler demonstrated, a few spherular elements lying scattered in the cytoplasm, most of them lying on the nuclear membrane (Plate 13, fig. 14). These elements vary in size and may be irregular in shape.

As the cell enlarges the Golgi elements multiply, and there is a gradual accumulation of them towards one side of the excentric nucleus (Plate 13, figs. 15, 16, 19). This localisation of the apparatus is never complete, for there is always a certain amount of Golgi apparatus material lying scattered away from the main mass.

The position of the yolk nuclear bodies can be observed except in the early stages by means of the negative pictures left on their solution (Plate 13, figs. 15, 16, 19). These "ghosts" are found lying either among the Golgi elements, or often just outside the main mass, in which case they are almost always surrounded by a ring of Golgi elements lying near together and fitting very closely on the surface of the "ghost." It is presumably these elements which Hirschler found and identified as mitochondria, and which led him to the erroneous conclusion that the mitochondria are derived from the yolk nuclear body.*

In this connection the observations recorded by Weiner (18) on Tegenaria may help to explain the apparent discrepancy between the present result and that obtained by Hirschler. Weiner found that the Golgi elements around the yolk nucleus developed inside them a granule which stained as did the yolk nucleus, and which finally became part of this latter. This suggests at once that Hirschler, in his preparations, had found these granules, but that the Golgi elements around them had not been impregnated, whereas in the material examined by the author, on ovaries treated by Da Fano's method, the granules are dissolved away, and the Golgi elements left.

The complex Golgi apparatus becomes more compact (Plate 13, fig. 19), but this lasts only for a very brief period. Then it commences to spread out again into the cytoplasm, forming at first a deep cap over the nucleus. It is just before and during this process that yolk formation commences to be visible in the egg.

As the elements spread out no interconnecting threads are found between them, but each is morphologically isolated from its fellows, and lies in cytoplasm free from other inclusions. The process of dispersion is most rapid in the neighbourhood of the nucleus, and tongues of Golgi bodies creep around its sides (Plate 13, fig. 20), eventually uniting on the far side to form a spherical or ovoid complex in which the nucleus lies excentrically. Meanwhile dispersal towards the periphery of the cell is taking place, the elements lying freely on the strands between the large vacuoles left by the solution of the yolk droplets.

The rapid increase in size of the apparatus leads to a decrease in size of its component elements, so that there appears a crescentic zone composed of many irregular granules of varying size, and a fair percentage of spherules all blackening strongly with silver. After this period of growth and dispersal the size of the elements becomes again uniform, and their shape more regularly spherical or corpuscular. They lie on the strands between the yolk droplets (Plate 13, fig. 21), and few, if any, signs of fusion with yolk were seen. The layer of peripheral cytoplasm sometimes has a fine argentophil dust scattered in it, but no signs of the larger Golgi elements can be found here, so that this region contains only the mitochondria.

No further changes have been observed in the Golgi bodies at the period when the nuclear membrane breaks down and the test-cells are cast out.

A word may be added about the appearance of the cell after various Golgi apparatus fixatives have been used. The description above applies chiefly to the appearance after Da Fano's technique. After Kopsch's unmodified method the cytoplasm is uniformly brown or yellow and quite often it contains large, watery-looking vacuoles. The complex Golgi apparatus is more easily impregnated than either of the diffuse forms, and sometimes consists of small, lamellar elements, as described by Hirschler, and sometimes of spheres. In either case it resembles that figured by Hirschler in the exactness of its localisation and in the lack of association of its elements with other cell inclusions (Plate 13, figs. 17, 18). The yolk, from its first appearance, is blackened, but in the early stages decolorises easily with turpentine. Later it becomes increasingly difficult to decolorise, over the entire cell, there being no especially blackened zone around the area occupied by the Golgi apparatus.

After Kolatschev's method, as has been described, the cytoplasm around the mitochondria as they spread out in the cell is full of small granules and filaments blackened by the osmic acid. The Golgi apparatus has not been observed in any of this material. To the end of yolk formation the blackened granules can be seen lying between the yolk spheres.

§ 6. *The Yolk Nucleus.*

The history of the yolk nucleus which Hirschler has described is as follows. It can be found in the youngest oocyte by staining with acid dyes, and soon becomes demonstrable without staining. The strong acidophil reaction which it exhibits indicates a cytoplasmic origin rather than a nuclear. There is usually only one yolk nucleus and never more than two. It has a sometimes deeply staining rim around a slightly less strongly staining interior. Around the periphery are a series of strongly stained acidophil granules, the mitochondria. These are developed from the yolk nuclear body, and pass out of it into the cytoplasm, and this causes the former to lose much of its staining power. Now the yolk nucleus becomes differentiated into a capsule with thick, sometimes two- or three-layered walls. This capsule may be closed or open at one side, but there are never any mitochondria in its interior. However, the Golgi apparatus may pass into the gap when the capsule is open. A stalk grows out from the capsule towards the nucleus. It reaches the nuclear membrane and acquires connection with the nuclear chromatin. Part of the substance of the nucleus passes up the stalk into the yolk nucleus. The latter reaches its maximum size at the time of maximum concentration of the Golgi apparatus. Thereafter it fragments, migrates peripherally among the yolk spheres and disappears altogether.

In the present material it has been found that in the very young oocyte where the cytoplasm is in the stage of primary oxyphily, the yolk nucleus is not always present (Plate 11, fig. 1A). In some cells it is to be observed as a very small acidophil granule lying closely apposed to the nuclear membrane. In other cells no sign of such a granule can be found, but these are comparatively rare. It can therefore be assumed that the yolk nucleus appears early in the oocyte, but that it is not passed on from the oogonium.

As the cell grows the yolk nucleus enlarges, and soon others begin to appear at points around the nuclear membrane (Plate 11, figs. 2, 3, 4). As the cytoplasm becomes strongly chromophil these granules take up acid dyes with even greater intensity than before. The number of yolk nuclear bodies is rarely confined to one or even two. There are more usually four or five irregular mammillated masses with a glassy appearance due to their high refractive index. In some cells there may be twelve or more of these masses forming an irregular girdle round the nucleus. This large number is exceptional, however, and may be due to early fragmentation.

Although these masses are as a rule homogeneous, yet sometimes they appear to have a granular or fibrous structure, so that the homogeneous form may be due to the fusion of a clump of granules or filaments. Sometimes also there is a large vacuole inside one of the masses (Plate 11, fig. 4), giving it a capsular appearance, which suggests the apparatus figured by Hirschler, but never are they in the regular form which the latter author has described.

The growth of the yolk nuclear matter reaches a maximum during the early stages of secondary oxyphily of the cytoplasm, and there begins a process of fragmentation and dispersal whereby the masses are reduced to smaller and smaller granules which scatter and finally disappear entirely before the formation of yolk has proceeded very far (Plate 11, fig. 5).

It is astonishing that Hirschler noticed only one or at most two yolk nuclei in the oocyte, and also that he described such an ordered series of changes as those summarised at the beginning of this section. In the half grown oocyte the most striking object in the cell is usually the ring of irregular masses of yolk nuclear matter around the nucleus, surrounded by a fainter cloud of mitochondria. No signs of any changes in the yolk nucleus such as those observed by Hirschler were found in the material studied, and there is never any observable connection between it and the nucleus.

§7. *The Test and Follicle-Cells.*

In the germinal epithelium the cells destined to form the test and follicle of the egg can be distinguished from the oocytes by the presence in their cytoplasm of a series of granules which are grey after osmication, and after mitochondrial fixation stain faintly with acid dyes. The cytoplasm does not stain, and from this cause these cells sometimes have a transparent, fragile appearance (Plate 11, fig. 1).

As the oocyte grows, several of these cells are found lying on and indenting its surface. They multiply amitotically and form a flat, squamous epithelium of uniform cells. The oocyte grows rapidly and the investing cells become stretched out to form a complete mantle over it. Smaller cells with a dense, darkly staining cytoplasm appear inside the follicle-cell layer. These are the test-cells which lie in the cytoplasm of the egg (Plate 11, fig. 5). The follicle-cells are formed from the cells of the epithelium by a process of swelling which seems to be confined largely to the cytoplasm, and which is due to the appearance of vacuoles close to the nucleus (Plate 11, fig. 5), and the enlargement of these to an enormous size, until they fuse to form one large ovoid droplet. This droplet fills the whole cell and almost obliterates the nucleus. The contents of the vacuole do not stain, but the outer membrane has a highly refractile appearance after mitochondrial fixatives, and either takes up no stain, in which case it appears bright yellow, or takes up toluidin blue strongly and appears deep purple. After anilin fuchsin and methyl green it is usually unstained or reddish. Later this material fills the centre of the vacuole and is absent from the periphery.

Meanwhile the test-cells multiply, still lying immediately inside the follicle-cell layer. Their cytoplasm becomes less dense in texture and granules appear which eventually fill the whole cell, almost obscuring the nucleus (Plate 11, figs. 5, 6, 7). After mitochondrial fixation these granules do not stain readily. After fuchsin and methyl green they are dull brown in colour, indicating a more or less amphophil reaction. As the oocyte enlarges and reaches a stage just prior to yolk formation the test-cells increase and, becoming distended with granules, acquire a spherical shape and form a practically continuous layer in the peripheral cytoplasm (Plate 11, figs. 6, 7). The granules at this stage are markedly osmiophil, which points to the presence of lipoid constituents; they tend to become more basophil in reaction.

Yolk formation commences in the egg and is accompanied by what seems to be a liquefaction of the droplets in the test-cells. These stain less strongly than before and become less in numbers but greater in size owing presumably to fusion. Gradually the cells become emptied, until they are left, at the end of vitellogenesis, as empty shells containing a nucleus and a few cytoplasmic strands enclosing large empty vacuoles (Plate 11, fig. 8). No trace of the materials of the granules can be found outside the test-cells whether outside the egg or in its cytoplasm. The disappearance of this material, however, concurrent with the formation of yolk in the egg suggests that material from these granules passes into the latter, and plays some part in the formation of yolk. Finally after the germinal vesicle of the egg has broken down, the test-cells are extruded, and come to lie between the chorion on which the follicle-cells are based, and the surface of the egg (Plate 12, fig. 9).

Meanwhile the follicle-cells swell enormously through the appearance of large, empty-looking vacuoles in the cytoplasm. The droplet of basophil substance described above retains its identity and lies eventually towards the periphery of the cell. The nucleus remains small and lies, an inconspicuous body, at the base of the cell. The cytoplasm is reduced to thin lamellæ between the vacuoles. At the time when the test-cells are thrown out of the egg the follicle-cells swell even more into long finger-like cells having a length almost equal to the diameter of the egg (Plate 12, fig. 9). These are joined by their broad bases to form a continuous follicle standing away from the egg surface. The cells are narrow cones whose tips swell to form a rounded distal end, in which lies the basophil droplet. Owing to the pressure of surrounding oocytes the follicle-cells often cannot expand as described above, and even in eggs lying towards the surface of the ovary the swollen follicle-cells are doubled upon themselves after swelling up.

In the living follicle-cell the basophil droplet at the tip takes up neutral red, brilliant cresyl blue and nile blue sulphate with avidity. Methylene blue stains it very faintly. With neutral red it stains a deep brown. Other parts of the follicle-cells remain unstained by any of the intravital stains used. In the extruded test-cells, on the other hand, numbers of large colourless vacuoles remain after the disappearance of the osmiophil granules. These take up the first three of the above-mentioned stains in a peculiar fashion. One or two small droplets of an intense red or blue colour, according to the stain used, appear after a short period, in each vacuole where they exhibit marked Brownian movement. Gradually more appear, until the vacuole is full of them, when they fuse into a droplet which fills the vacuole.

§8. *The Yolk.*

It is not intended here to review the evidence upon the mode of formation of the yolk, but merely to record changes in the latter during and after its formation. As it is formed the yolk has an acidophil reaction. After mitochondrial fixation, in unstained cells it is present as pale brown or yellow spheres of various sizes. These stain fairly strongly, and at first uniformly, with anilin fuchsin and iron hæmatoxylin. Although the intensity of staining is uniform for each granule it varies considerably among the granules as individuals; some stain strongly, some weakly, and there are all grades between these two extremes. After Bouin, Formol, Carnoy, Smith, etc., the yolk droplets are dissolved, leaving only alveolated cytoplasm. After osmic methods the yolk blackens, but is at first easily decolorised by turpentine, later becoming increasingly resistant to this reagent.

As the egg approaches the time when the germinal vesicle will break down, the yolk shows signs of being no longer homogeneous. Scattered among the uniformly stained spheres are droplets in which the chromophil substance is reduced to scattered granules occupying a varying proportion of the droplet, the rest of which is unstained. This is more evident after the germinal vesicle has disappeared. At this time also there appears a zone, usually at first located towards one side of the egg, in which the yolk is pale grey and lies in colourless vacuoles in the cytoplasm. Before the egg is passed out of the ovary this zone moves into its centre. At first this was considered to be due to lack of penetration of the yolky egg by the fixative, but frequently eggs were found in which the normal deeply stained droplets were scattered among these watery yolk droplets, and also the zone was found in the majority of the eggs fixed after being taken from the oviduct and left in the fixative for almost as long a time as were the whole pieces of ovary (Plate 12, figs. 10 and 11). In the larva pale droplets can be found scattered among the darkly staining granules of some of the cells, especially those of the endoderm. Consequently this zone cannot be regarded as an artefact due to lack of penetration, but must be treated as a definite region of the egg.

After the germinal vesicle has burst the cell has a much denser appearance; as has been recorded above, and this is in part at least due to an increase in the staining powers of the yolk droplets, except in the pale watery zone which has just been described.

§9. *The Cytoplasm.*

There is little fresh to record about the cytoplasm. It passes through the three stages described by Hirschler, a primary stage of oxyphily, corresponding to the primary diffuse condition of the Golgi apparatus, a stage of basophily which corresponds to the stage of complexity of the apparatus, and a secondary oxyphil stage when the Golgi apparatus is in the secondary diffuse stage.

When the germinal vesicle breaks down the material of its interior may appear as a cap of cytoplasm freer from yolk droplets, below the mitochondria of the vegetal pole; but, on the other hand, the breakdown is accompanied by a shrinkage of the egg, and this suggests that fluid is being expelled from it. The cytoplasm beneath the mitochondrial layer is freer from yolk droplets than is the centre of the egg and the animal pole (Plate 12, fig. 9); but this may be due, quite probably, to a slight rearrangement of the inclusions, with a tendency towards concentration of the yolk in the animal hemisphere. In all probability the nucleoplasm, on its release from within the bounds of the nuclear membrane, mixes with the cytoplasm. This would disturb the equilibrium between the egg and the external medium, leading to an expulsion of fluid from the former, to restore the balance.

§ 10. *The Nucleus.*

No observations on the chromatin content of the nucleus in relation to chromosome formation and the maturation phenomena were made.

In the young oocyte (Plate 11, figs. 1, 2) the nucleus is a relatively large clear vesicle containing a fairly large acidophil nucleolus, which is homogeneous in texture and regularly spherical in shape. It lies close to the nuclear membrane. As the oocyte grows the nucleus enlarges, but less rapidly than the cytoplasm and the nucleolus, which latter soon becomes relatively enormous.

In "non-mitochondrial" preparations no changes in the staining affinities of the karyoplasm are observable. The nucleus enlarges progressively as a regularly spherical or ovoid body, and a heavy, irregular, acidophil granulation becomes more and more apparent. Meanwhile the nucleolus becomes vacuolated to such an extent that finally it is packed with small vacuoles, some with a refractive index higher than that of the nucleolar material, some whose index is about equal to that of the latter.

Following mitochondrial fixatives the nucleus, after a period of primary achromasy, passes into a stage of acidophily (Plate 11, figs. 3, 4, 5, 6, 7), during which it often stains uniformly almost as deep a red as the nucleolus with anilin fuchsin, and a deep blue with hæmatoxylin. At the same time the nuclear membrane is irregular, running out into long lobes (Plate 11, fig. 7), with correspondingly deep indentations between them. The nucleo-cytoplasmic ratio is being disturbed by the rapid growth of the cytoplasm, and no doubt the irregularity of the nuclear surface is due to an attempt to restore the equilibrium by increasing the nucleo-cytoplasmic surface.

In material stained with iron hæmatoxylin the nucleolus remains a uniform deep black throughout the oogenesis, but after anilin fuchsin a certain amount of highly refractile vacuolation can be observed as in the " non-mitochondrial " material.

In Da Fano material the nucleolus stains at first an extremely pale uniform lilac in Ehrlich's hæmatoxylin (Plate 13, figs. 14, 15). As it increases in size an increasing amount of argentophil granulation appears. This probably represents the highly refractile vacuolation observed in other material. A little later, paler vacuoles appear having a refractive index equal to that of the darker material. Gradually these paler vacuoles occupy the major part of the nucleolus, sometimes as two or three vacuoles, more often as one large one which leaves but a thin crescent of the granulated material over one side (Plate 13, figs. 16, 20, 21).

After the commencement of yolk formation the nucleus regains its regular shape and the karyoplasm loses its acidophility and homogeneity. The greater part of the nucleus, in section, is now occupied by a faintly acidophil, granular precipitate. The nucleolus lies excentrically and is large and strongly acidophil in its reactions.

When yolk formation is at an end the nucleolus fragments into spherical droplets which lie scattered in the nucleus until its membrane breaks down, when they pass into the cytoplasm and become lost to view, together with the karyoplasm. The nucleus becomes reconstituted as a much smaller body and enters upon the early stages of the first maturation division. Before the egg leaves the ovary, the spindle is formed and the chromosomes are arranged on the equatorial plate (Plate 12, fig. 13). The nucleus remains thus, in metaphase with the spindle axis parallel to the surface of the egg until the entry of the sperm. No attempt was made to follow the nuclear changes during the period between the break down of the germinal vesicle and the formation of the metaphase spindle, owing to the difficulties experienced in finding and identifying the nucleus among the yolk droplets, and also to the fact that these nuclear changes do not bear upon the problem under investigation.

Evidently there is a considerable amount of variation in the state of the nucleus at the times when the egg leaves the ovary and is fertilised, for Conklin (3, 4) has recorded the breaking down of the germinal vesicle as occurring normally in the oviduct, after the egg has left the ovary, and Duesberg (9) has recorded the nucleus as being only in early prophase at the time of entry of the sperm, whereas the above observations record the formation of the metaphase spindle before the egg has left the ovary.

§ 11. *Changes after the Egg has left the Ovary, and on Fertilisation.*

After the egg has passed into the oviduct, the concentration of the mitochondria into a cup over one half of the egg gradually becomes more marked. At the same time the yolk spheres become densely packed in the animal hemisphere, while beneath the mitochondria in the vegetal hemisphere they are loosely packed (Plate 12, figs. 9, 10). The zone of unstained yolk lies centrally now.

The outline of the egg becomes irregular again, probably because of a further shrinkage in size which causes the surface to be thrown into wrinkles in order to adjust itself to this change. Here the mitochondria of the cup are closely packed in the cytoplasm up to the surface of the egg (Plate 12, figs, 9, 10). There is no sign of the indentation of the outer surface of this zone, which has been recorded by Meves (14) and Duesberg (8, 9) for the mitochondrial crescent after fertilisation. The thickness of the cup is not great, and the inner surface is more sharply defined than it becomes later, as there are less mitochondria scattered loosely among the yolk granules immediately below it. There are a few here as there are among the yolk granules all over the cell, but they are insignificant compared with the number present after fertilisation.

In sea-water the eggs again decrease slightly in size, their surface becomes regular once more, and their shape spherical or more often ovoid. The zone of protoplasm beneath the mitochondrial cup, where the yolk was small in amount, disappears almost entirely. Sometimes a narrow, more loosely packed band can be found, but more often the yolk extends as a dense band right up to the mitochondria.

The movements of the egg cytoplasm, described by Conklin (3, 4) have been denied by Duesberg (9), who states that there is no difference in the zonation of the egg substances before and after fertilisation. While the present results tend to confirm Duesberg's claim, they yet show that there is a considerable difference in the degreee of localisation in the egg after the entry of the sperm. Then the mitochondria become concentrated into a thicker shallower cup with less clearly defined limits than before. This is due, partly, to the addition of fresh granules by migration, and partly to looser packing in this region (Plate 12, figs. 11, 12).

Meanwhile among the yolk droplets, all over the egg, spherical areas appear whose limits are defined by the yolk, and within which no inclusions of any sort can be found (Plate 12, fig. 12). They vary in number up to about fifteen, and they seem to be orientated roughly about a point near the centre of the mitochondrial cup. Appearing almost immediately after fertilisation, they are very prominent in the cell for about twenty minutes, after which they have disappeared entirely.

At the same time that the above phenomenon is observable, the mitochondrial cup becomes deeply indented all over its outer surface, and the bays are filled with clear protoplasm, as has been recorded by Meves (14) and Duesberg (9). Immediately inside the mitochondrial area is a thin band of yolk droplets, and inside this again, an area where the yolk is small in amount and loosely packed. The animal hemisphere remains full of closely packed yolk. Evidently there is a process of concentration of the cytoplasm in the vegetal half of the cell, and the patches of clear cytoplasm which appear amongst the yolk droplets are indications of its movements. What the physical cause of this is has not been determined.

The changes undergone by the sperm have not been followed, but the observations on the paths of the pronuclei and the distribution of materials during development have agreed very closely with those of Duesberg, and a repetition of the details of the embryology would only be redundant.

§ 12. *Discussion.*

The form and consistency of the Golgi apparatus is a problem which is engaging the attention of cytologists, the more since the recently published work of Parat and his colleagues thereon. Parat (15) claims, after study of cells intravitally stained with neutral red, that the Golgi apparatus of animal cells is the homologue of the vacuolar system in plant-cells, and that its form is essentially vacuolar. The action of fixatives on this and the surrounding tissues produces the reticular pictures so characteristic of the Golgi apparatus of many cells.

On the other hand Bowen (2) has suggested recently that the form of the Golgi apparatus is of secondary importance, the essential thing being its presence in the cell. He considers that, in germ cells at least, it is composed of two materials, one forming the Golgi apparatus proper, the other the idiosome, or archoplasm of many workers, and he suggests that this may be so in all cells.

Although Parat's work has been questioned by many workers yet his results are extremely suggestive. There are several reasons why the Golgi apparatus might be expected to be vesicular in nature. Firstly the cell is a fluid or semi-fluid system, and therefore any fluid bodies lying in it will either be dispersed in the mass of the cytoplasm for lack of any cohesive force to hold their molecules together, or else they will assume a shape as nearly spherical as possible on the principle of conservation of energy. There is no evidence that the Golgi apparatus, at least in its complex form, is solid or even semi-solid. On the contrary what little evidence we have points to the absence of a solid network in the living cell comparable with the reticular apparatus, and the maintenance of a fluid network would necessitate the expenditure of enormous energy on the part of the cell.

Secondly the scattered type of apparatus characteristic of many cells is composed of elements which are probably vesicular, though a plate- or rod-like form has been advocated. This type is primitive, and from it the reticular structures have been shown to arise, and *vice versa* (*cf.* Hirschler (12)). Hence the structure of the reticular apparatus is similar to that of the scattered apparatus, and so is probably vesicular.

Lastly, the vacuolar system of plant cells has been suggested as the homologue of the Golgi apparatus of animal cells, which implies a similar structure of both organs. Bowen has questioned this on the grounds mainly of the lack of specificity of Golgi apparatus techniques in plant cells. He suggests that the homologue of the Golgi apparatus may be one of the two types of chondriosome in the plant cell, one of which forms the plastids, the other remaining unchanged. Now, in the eggs of Ciona there are two types of chondriosome, one, comparable to that forming the plant plastids, swells to form yolk, the other remains unaltered. But there is also a typical Golgi apparatus, and hence the homologue of the Golgi apparatus must be sought otherwhere than in the chondriosomes of the plant cell.

The vesicular form of the mitochondria and their change to granular form on the extrusion of the test-cells has already been mentioned. The reason for the change is obscure, but it may possibly be that the mitochondria share in the general shrinkage brought about at this period, and this causes the assumption of a granular form.

In regard to the yolk nucleus, work is being carried out which it is hoped will establish the identity of this organ in a cell by means of its mode of formation. Until this has been done the somewhat chaotic state of the literature on the subject renders discussion somewhat difficult as so many different bodies in the cell have been labelled yolk nucleus without due regard to their actual structure and homologies.

Turning now to the question of yolk formation. There seems to be a general agreement among workers on Ascidian oogenesis that yolk arises from the mitochondria. The only dissentients are Crampton, who finds that the yolk of *Molgula* arises by the swelling up of the yolk nuclear granules, and Loyez, who derives the yolk of *Ciona* from the mitochondria but not that of *Cynthia*. The earlier workers have sometimes failed to identify the mitochondria as such, owing to the unsuitable techniques used when their work was carried out, and also to the fact that these bodies were not so well known as they are now. But, from the history of the bodies described as giving rise to the yolk, they can now be identified with a fair degree of certainty as mitochondria.

There are three possible alternatives to be considered as to the part played by the mitochondria in yolk formation. In the first place there may be direct chemical transformation of the material of the mitochondria into yolk material. Secondly, there may be deposition of yolk inside the mitochondria, its material being derived from the cytoplasm under the influence of the mitochondria. Lastly, there may be deposition of molecules of mainly protein composition among the molecules that compose the mitochondrial material, and the complex so formed is then yolk.

The mitochondria and true yolk are very closely allied chemically, each having a protein and lipin composition, so that no difficulty is experienced in conceiving of direct chemical transformation. On the other hand, the mitochondria are such widely distributed and omnipresent materials in cells that great caution should be used in postulating the loss of this material by chemical changes. Besides this, in the particular case under discussion there are other factors which render the situation more complex.

The materials used by the mitochondria in yolk formation must be derived from the cytoplasm in the form of solutions, since there are no visible evidences of their presence around the mitochondria, in sections. There are two probable sources from which they may be derived. In the first place the yolk nuclear material disappears into the cytoplasm just at the period when yolk commences to be formed. Secondly, the test-cells become emptied of their granular contents during this process. The materials derived from these sources are then present in large amount in the cytoplasm in the early stages of yolk formation. Unfortunately no evidence can be found of their presence in, or absence from the cytoplasm either before or after the latter process, but the coincidence of their disappearance at the time when the mitochondria begin to swell, suggests that they pass into the latter, and are there compounded or mixed into yolk, together with the material of the mitochondria themselves, after the manner suggested by Cowdry (6), in his exposition of the surface film theory.

The origin of the lipoidal granules of the test-cells has not been discovered, owing to the small size of the latter, and to the large amount of material present, which obscures completely any processes going on in them. The yolk nuclear material however, after a short period when its relations have not been minutely observed, is found to be in intimate contact with the Golgi apparatus of the oocyte. It can with sufficient justification be assumed that this relation exists in the first stages of its evolution in the cell as well as at this later period. The author has been strengthened in his opinion by the recent work of Weiner (18), published after the above conclusion was arrived at, in which the yolk nuclei of *Tegenaria* and *Lithobius* have been found to be secreted by the Golgi apparatus from the moment of their appearance in the cell. As the Golgi apparatus reaches its maximum size and concentration the amount of yolk nuclear material also grows very large. This, coupled with the striking "palisades" of Golgi elements investing outlying portions of the yolk nucleus, is very strong evidence that the latter are secreted by the Golgi apparatus, in just the same way as are, for instance, the secretion granules of the goblet cells of the intestine.

In a recent note Parat and Bhattacharya (16), working with vital stains, have claimed to have confirmed Hirschler's account of the oogenesis of *Ciona* in all except that part relating to the yolk nucleus. On this point they state that they could not demonstrate this organ by the methods used, and after a series of experiments the details of which they do not record they conclude that it is an artefact. In the circumstances it is useless to argue the point. It is highly improbable, however, that an object which stands out with such startling clearness in all preparations, and reacts specifically to various reagents and dyes, should be an artefact.

The nucleolus plays no visible rôle in the growing oocyte. It is very large and becomes highly vacuolated, with two categories of vacuole, one of high, the other of low refractive index. There is no sign of fragmentation of the nucleolus until the germinal vesicle is about to break down, nor is there any nucleolar extrusion into the cytoplasm. The vacuolation is a sign of activity in the nucleolus, but the relation of this activity to that of the cell cannot be determined.

The process of yolk formation, then, in *Ciona* seems to be this: into the cytoplasm of the growing oocyte are poured two materials in solution, a lipoid constituent from the granules of the test-cells, and an albuminous material secreted by the Golgi apparatus. The molecules of these two compounds become aggregated around and finally inside the mitochondria, which are scattered in the cell. Here they are compounded or mixed into yolk, the material of the mitochondria taking part in the formation of the former, but possibly retaining its identity as a material.* The primary origin of the constituent derived from the Golgi apparatus may quite conceivably be the nucleolus, whose vacuolation may be the outward and visible sign of an invisible passage of its material through the nuclear membrane to the cytoplasm, where the Golgi apparatus converts it into visible form once more. The similarity in staining affinities and refractive index of the nucleolus and yolk nucleus, and also the aggregation of the Golgi apparatus, as is very often the case, near the nuclear membrane, lend a certain amount of colour to what is admittedly a speculation.

§ 13. *Summary.*

1. The previous accounts of yolk formation are reviewed briefly.
2. In *Ciona intestinalis* the mitochondria are present as chromophobe vesicles with acidophil auræ in the youngest oocytes. They increase in number enormously, and all except those in the narrow peripheral layer, and a few scattered individuals, swell to form yolk. After the extrusion of the test cells the remaining mitochondria become granular and uniformly acidophil. The peripheral layer forms a deep cup, which becomes shallower and thicker at the time of fertilisation.
3. The Golgi apparatus passes from primary diffuse to complex, and then to secondary diffuse stage. It consists always of small argentophil vesicles and irregular masses.
4. It is suggested that the " yolk nucleus " is secreted by the Golgi apparatus very early in oogenesis. It fragments and becomes dissolved in the cytoplasm.
5. The test-cells pour lipoid materials into the egg during yolk formation.
6. The cytoplasm passes from primary oxyphily to basophily and then to secondary oxyphily. These changes are correlated with changes in the Golgi apparatus, as has been described by Hirschler.
7. The germinal vesicle bursts and the reconstituted nucleus reaches the metaphase of the first maturation division usually before the egg leaves the ovary.
8. A suggestion as to the method of yolk formation in *Ciona* is made (see Discussion, p. 158).

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* In this connection the observations by Coghill on the living cells of embryonic Amphibians are interesting as a record of the origin of mitochondria from yolk, in the manner which the above theory would seem to explain. (Coghill, G. E., 1915. Preliminary studies in intracellular digestion and assimilation in amphibian embryos. " Science," N.S., vol. 42, pp. 347–350.)

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15. EXPLANATION OF PLATES

LETTERING OF PLATES

CA., Clear areas in yolk.
FC., Follicle-cells.
FMC., Follicle mother cells.
GA., Golgi apparatus.
GB., Golgi bodies.
M., Mitochondria.
PS., Polar spindle.
PY., Pale staining yolk.
TC., Test-cells.
YD., Yolk droplets.
YN., Yolk nucleus.
YNG., Yolk nuclear " ghosts."
YV., Vacuoles left on solution of yolk.

PLATE 11.

FIG. 1.—Young oocytes in the germinal epithelium, showing few vesicular mitochondria and absence (*a*), or very small size, of the yolk nuclear granules. Champy-Kull. Mag. *ca.* 1500.†

FIGS. 2, 3, 4.—Larger oocytes showing increase in numbers of mitochondria, and growth of yolk nucleus. Champy, AFMG.* Mag. *ca.* 1500.†

FIG. 5.—Older oocyte showing later stage in break up of yolk nucleus. Kolatschev, AFMG.* Mag. *ca.* 800.

FIG. 6.—Oocyte showing commencement of formation of yolk. Kolatschev, AFMG.* Mag. *ca.* 1000.†

FIGS. 7, 8.—Later stages of yolk formation. Champy, Fig. 7, IH.* Mag. *ca.* 1200. Fig. 8, AFMG.* Mag. *ca.* 700.

PLATE 12.

FIG. 9.—Oocyte after breakdown of germinal vesicle, showing also test- and follicle-cells. F.w.a.,* IH.* Mag. *ca.* 400.

FIG. 10.—Oocyte from oviduct. Kolatschev, IH.* Mag. *ca.* 700.

FIG. 11.—Oocyte immediately after fertilisation, showing beginning of formation of crescent and turning of polar spindle. Champy, IH.* Mag. *ca.* 700.

FIG. 12.—Fertilised egg showing development of spaces among yolk droplets (see text) and fully formed cup of mitochondria. Nassonov, AFMG.* Mag. *ca.* 700.

FIG. 13.—Polar spindle of ovarian egg to show aggregation of mitochondria about poles. Champy, IH.* Mag. *ca.* 1500.†

PLATE 13.

FIG. 14.—Young oocyte in the germinal epithelium to show distribution of Golgi elements. Da Fano. Mag. *ca.* 1500.†

FIGS. 15, 16.—Older oocytes to show yolk nuclear " ghosts " among aggregated Golgi bodies. Da Fano. Mag. *ca.* 1300.†

FIGS. 17, 18.—Two oocytes to show appearance of Golgi apparatus after Mann Kopsch. Mag. *ca.* 1300.†

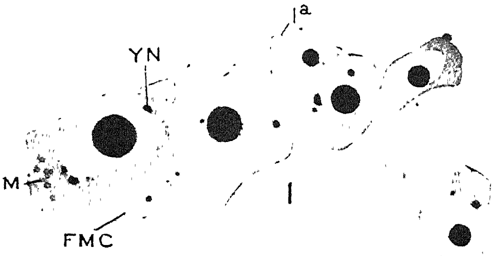
FIG. 19.—Complex stage of Golgi apparatus in half-grown oocyte. Da Fano. Mag. *ca.* 1000.†

FIG. 20.—Dispersal of Golgi elements in oocyte following yolk formation. Da Fano. Mag. *ca.* 700.

FIG. 21.—Full grown oocyte just before breakdown of germinal vesicle, to show uniform distribution of Golgi elements except at periphery. Da Fano. Mag. *ca.* 800.

* AFMG.—Anilin fuchsin followed by Methyl green.
F.w.a.—Flemming's solution without acetic acid.
IH.—Iron Hæmatoxylin.

† These figures were made with a one-sixteenth fluorite objective.



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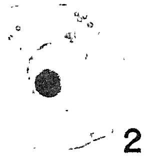

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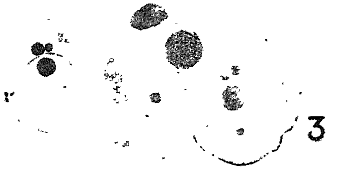

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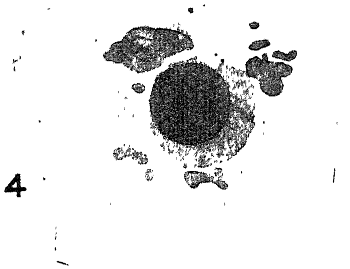

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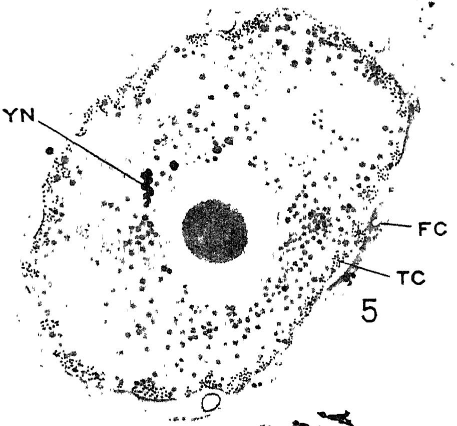

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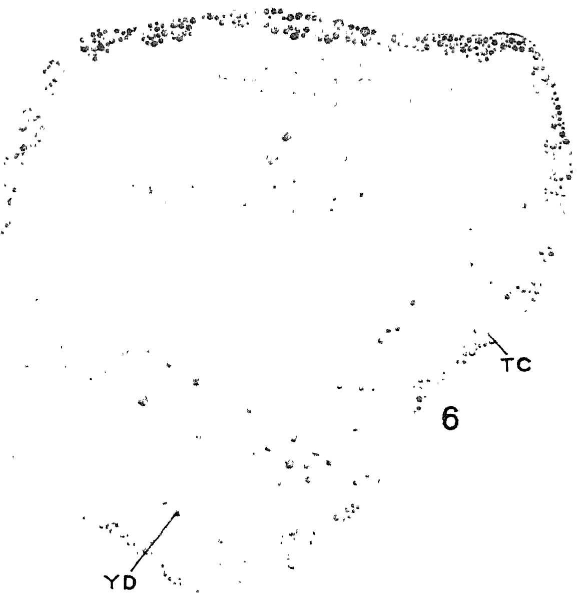

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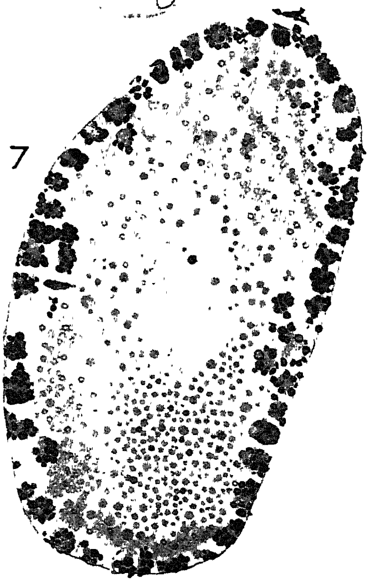

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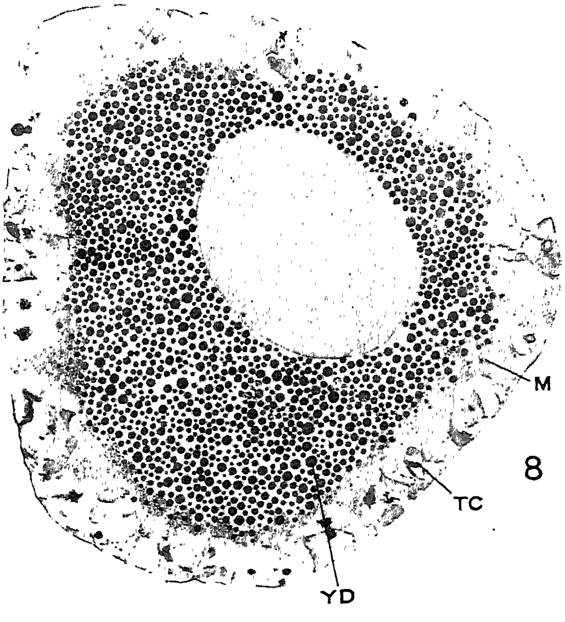

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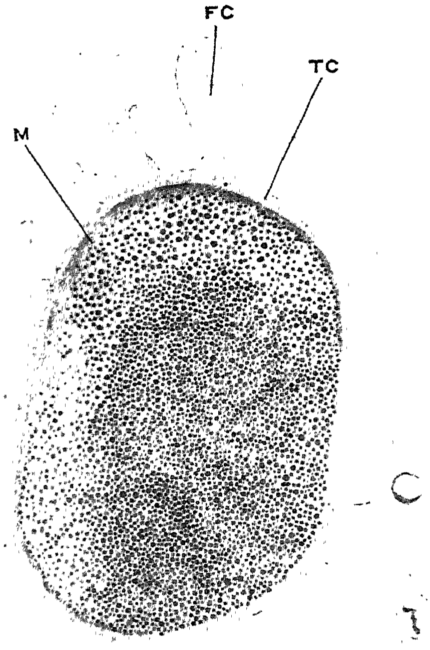

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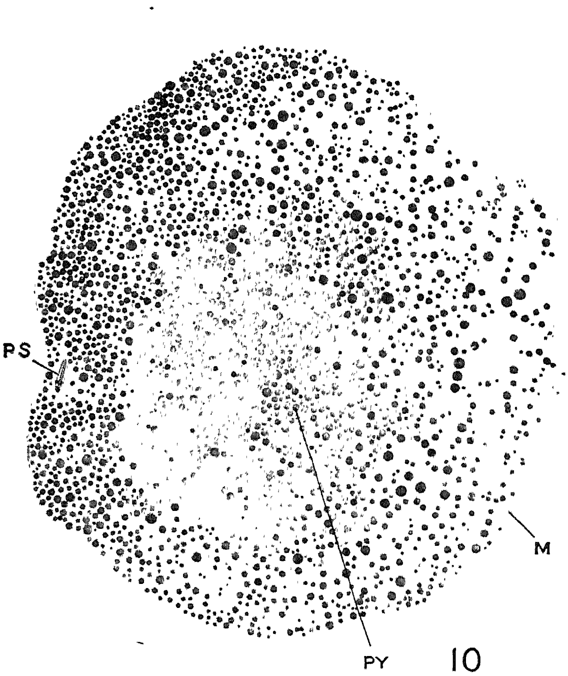

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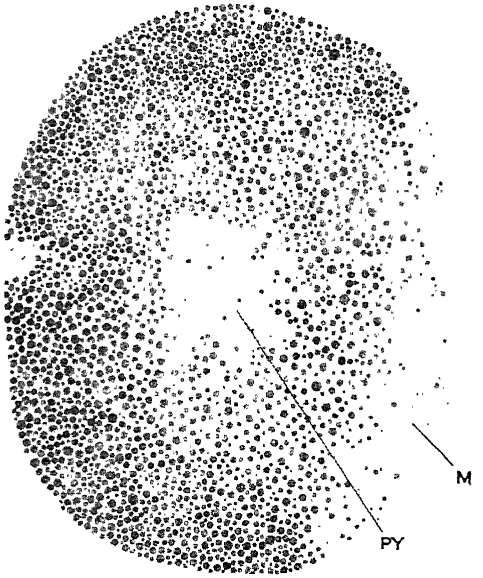

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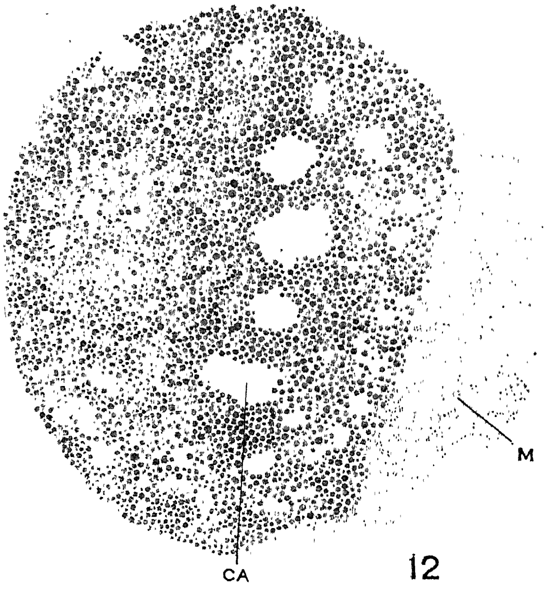

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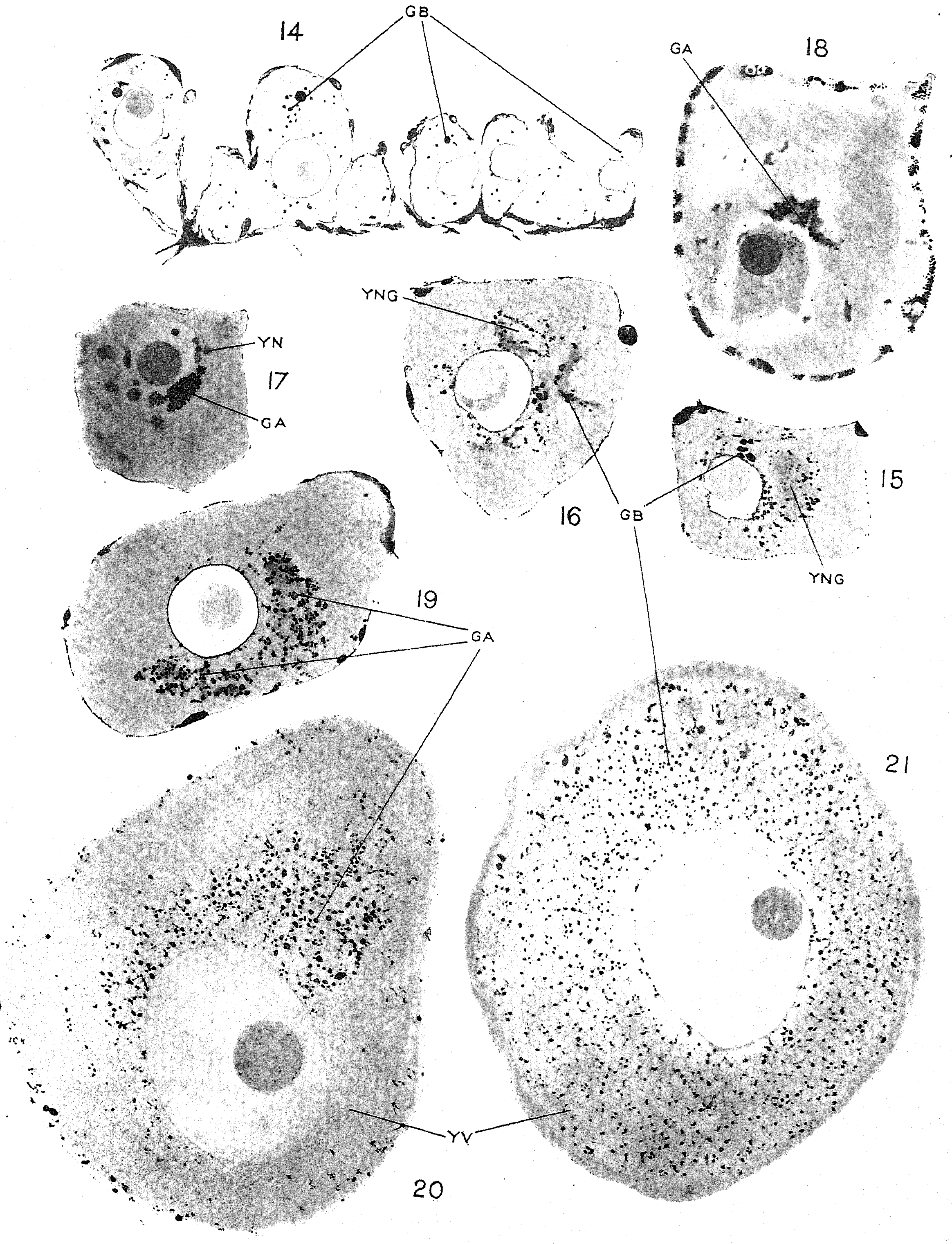
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*Basal Metabolism as Determined by the Respiratory Exchange.**

By F. A. PICKWORTH, M.B., B.S. (Lond.), B.Sc. (Lond.), A.I.C.

(Communicated by Dr. J. S. Haldane, F.R.S.—Received December 8, 1926.)

PART I.

Recent work upon mental disorders has shown that there are important changes in the endocrine organs as well as in the central and sympathetic nervous system. Changes have been especially studied in the sex glands and in the thyroid; and it seemed evident that the determination of the basal metabolic rate of mental hospital patients might do much to add to our knowledge of disorders of the working of these glands. The present research was undertaken under the direction of the late Sir Frederick Mott, who was Honorary Director to the Joint Board of Research for Mental Diseases, City and University of Birmingham.

From the beginning it was evident that the usual Douglas bag or spirometer methods, involving mouth and nose pieces or a mask, were quite unsuitable for the work in hand, it being absolutely necessary that the disturbing factors of fear, anxiety and excitement should be eliminated. A chamber was, therefore, constructed similar to one which Prof. J. S. Haldane uses for various experimental purposes, in which the patient could sit, lie or sleep without disturbance for a period of two hours, the metabolism being measured by determining the changes in the air of the chamber at the end of this period. As, however, these changes are small, a special modification of the Haldane air analysis apparatus was required.

Description and Construction of Apparatus.

The chamber used was modelled upon that used in the Mining Research Laboratory of Birmingham University, and consisted of a room 6 feet 7 inches high, 6 feet 3 inches wide, and 8 feet 9 inches long, having a capacity of over 10,000 litres. This room was lined with thick lead, all joints being soldered, the screw heads being also soldered over to make the walls completely airtight.

[* Shortly before his fatal illness Sir Frederick Mott told me that the accompanying paper by Dr. Pickworth was approaching completion, and that he hoped to present it to the Royal Society. He also asked me to read it carefully before it was sent in. The work is on the lines of many other investigations inspired or carried out by him, and its publication is a fitting reminder of his great services to Medicine and Physiology.—J.S.H.]

The roof was of internally wired glass; there was a large window for observation, and the door was wide enough to admit a bed. An electric fan set at a suitable inclination worked continuously to mix thoroughly the air, and warming or cooling was effected by water in copper pipes. The room was pleasantly decorated, the walls painted and surmounted by a picture rail and a frieze. The complete chamber appeared a good substitute for a light airy cheerful bedroom. This chamber was built in a room at Hollymoor Mental Hospital, within easy access of both Male and Female Wards.

There were many constructional details to be dealt with before the chamber was satisfactory for use. Especial difficulty was encountered in obtaining an air-tight seal around the door. Soft rubber contact was insufficient and various devices of plasticine, softened plasticine and vaseline seals were likewise useless. A satisfactory result was at last obtained by the use of dovetailing iron frames—a trough on the door, the bottom of which had a bed of plasticine of depth exactly equivalent to the iron frame on the chamber, and between the frames in the trough of the door frame, a continuous rubber tube containing air which became indented when the door was closed. This proved quite satisfactory, allowing no escape of air at all.

A further difficulty was encountered in the construction of the roof. The testing of the chamber was made by heating the air 2 or 3° and measuring the rate of leakage. Since the volume of the chamber was 10,000 litres an increase in temperature of 3° C. meant an increase in volume of 100 litres, *i.e.*, 1 per cent. The upward pressure upon the roof equalled, therefore, 1/100 by 15 by 8,000 lbs., *i.e.*, considerably over half a ton, which proved too much for the framework, with the consequence that the roof lifted, allowing escape of air. Similarly a negative pressure caused by a fall of 3° C. was sufficient to lift the lead floor visibly. This difficulty was finally overcome by additional screwing-down of the roof, and a device by which the leakage was measured and could be allowed for if necessary. The pressure within the chamber was never allowed to exceed one-half per cent., *i.e.*, 50 litres, and the final total leakage was infinitesimal.

The indicators used for the chamber consisted of:—

1. A bulb (see fig. 1, A), within the chamber, having a tube dipping in a coloured fluid outside, which showed temperature changes within and barometric change outside chamber (but was not affected by pressure changes within the chamber).
2. A bulb B, within the chamber, connected by a tube dipping into fluid: the air above which was in communication with the interior of the chamber and showed changes in pressure that were not due to temperature, *i.e.*, measured any leakage. It was unaffected by temperature changes within the chamber or by barometric change without. The amount of leakage in litres could be seen at a glance during any part of the experiment. Twenty-five litres caused a change of 1 cm.

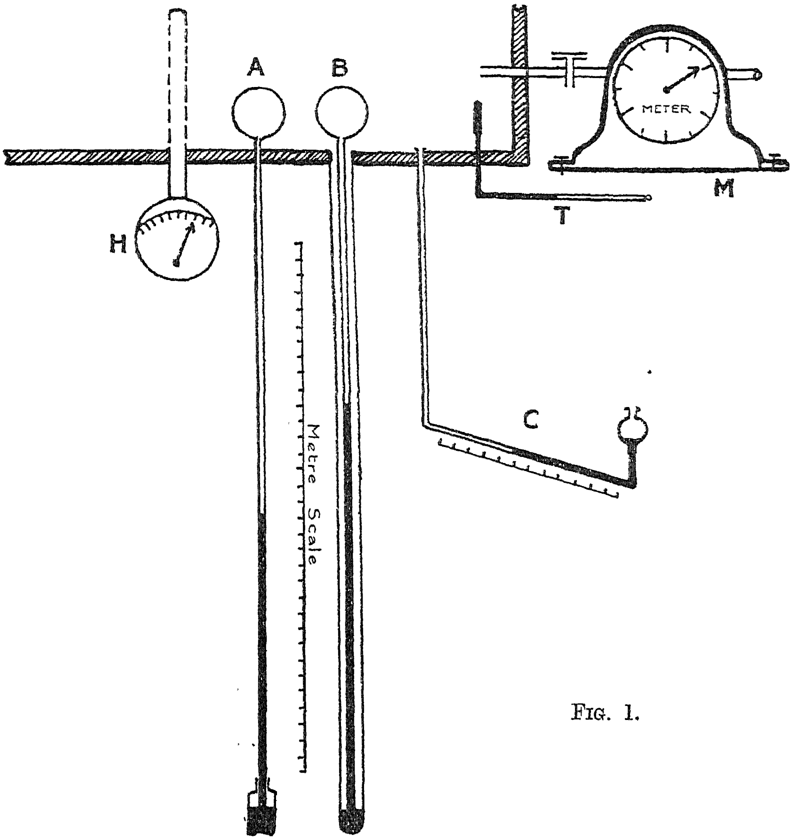


FIG. 1.

3. C, a simple pressure-gauge, indicating relative pressure within and without the chamber.
4. A gas meter used for testing the chamber.
5. A thermometer T registering to 1/10° C.
6. An Edney hair hygrometer H inserted through the chamber wall near the fan.

By pumping air into the chamber through the meter the indicators were tested and the leak measured. It was found that one large division of indicator C equalled 5 litres, whereas in B and C each 0·2 c.m. was equivalent to 5 litres. The total combined leakages from the whole chamber were on testing shown to be reduced to the equivalent of a single pinhole.

Capacity of chamber—

Height, 202·5 cm.	= 10,114 litres
Length, 261·5 cm.	
Width, 191·0 cm.	
Recess, 68 cm. by 190 cm. by 3 cm.	= 30·8
,, 104½ cm. by 190 cm. by 5 cm....	= 99·3
	10,252 gross.

less picture rail 2·0, window frame 1·2, fan and bulbs 1·7, top beading 12·6, hot-water pipes 8·7, baseboard 29·7, chair 67·2, two pillows 3·5, blankets 1·2, footrest 1·0, table 7·0, patient 64·0 = 200 litres.

Net volume of chamber, when containing patient and furniture, say, = 10,050 litres.

The above result was checked by decomposing 200 gm. of 97·8 per cent. $NaHCO_3$ within the chamber, by adding acid, this gave an increase of 0·534 per cent. CO_2.

The total CO_2 available from the bicarbonate, at room temperature and pressure is—

$$\frac{200 \times 97{\cdot}8 \times 22{\cdot}2 \times 760 \times 294}{100 \times 84 \times 730 \times 273} = 58 \text{ litres.}$$

As possibly not all the 58 litres would be set free immediately from the reaction mixture, the capacity calculated, $\frac{58 \times 100}{0{\cdot}534} = 10{,}860$ litres, is about one-half per cent. too high, but near enough to check the capacity satisfactorily.

Carbon dioxide from a cylinder was introduced into the chamber through a Siebe-Gorman gas meter. The meter reading was 52·5 litres. The actual amount would be slightly greater at the temperature and pressure of the chamber.

Analyses of the gas gave the following results: (1) 0·523, (2) 0·522, (3) 0·525, (4) 0·525 per cent. CO_2. After 48 hours (there being no control of temperature variation of the chamber): (1) 0·518, (2) 0·514, (3) 0·518. After 4 days: (1) 0·506, (2) 0·503.

The above forms an additional satisfactory check upon the capacity of the chamber and demonstrates the absence of important leakage.

Apparatus used for the Gas Analysis.

The apparatus used was a Haldane-type air-analysis apparatus, modified to use 100 c.c. for each test. It was first fixed direct upon the chamber, but this made it impossible to check the results by duplicate analyses. The apparatus was, therefore, transferred to the laboratory; samplers being used to transfer the gases, consisting of a 300-c.c. cylinder with capillary taps at each end and connected with a mercury reservoir. The samplers were rinsed with acid just before use.

Theoretical Considerations.

In addition to the usually compensated pressure-temperature factors the following are of importance in order to obtain an accuracy of 1 per cent. absolute.

Readings must be within 0·003 c.c. on 100-c.c. sample, since the usual CO_2 excretion in the chamber for 2 hours amounts to about 0·3 per cent.

The meniscus of the adjustment tube must be within 0·3 mm., an amount easy to obtain with the fine line on the mirror as described.

The difference in volume of tubing of burette and control not actually under water must not exceed 1/6 c.c. The calculated maximum change of temperature, during which vapour tension does not alter, is limited to 0·02° C. In order to overcome this important source of error fine cotton strands were placed in the control burette, dipping into 5 per cent. sulphuric acid, as shown in fig. 2.

The absorption of gases by the reagents employed is negligible with the smaller apparatus, but owing to the time necessary to establish equilibrium in the larger apparatus and the higher accuracy desired, it is imperative that only small volumes of reagents be employed. Actual calculations based upon solubility of the gases show that the maximum amount of fluid to moisten gas must be under 0·6 c.c., and the amount of potash for absorption, assuming a temperature variation of 1° C. during experiment, is 4 c.c. Similarly since gas dissolved under pressure is not instantly quantitatively released the pressure changes must never be large.

These factors are eliminated by using the small amounts of liquid reagents, and suitable slow raising and lowering of the reservoirs. The reagents were frequently renewed, and a temperature of about 18° C. maintained in the water-baths.

Apparatus.

Having all the above facts in mind the gas analysis apparatus was entirely re-designed, in order that the allowed error should never be reached, and in most cases reduced to half or even less. An apparatus was, therefore, constructed as illustrated (fig. 2), the practical details of which were as follows :—

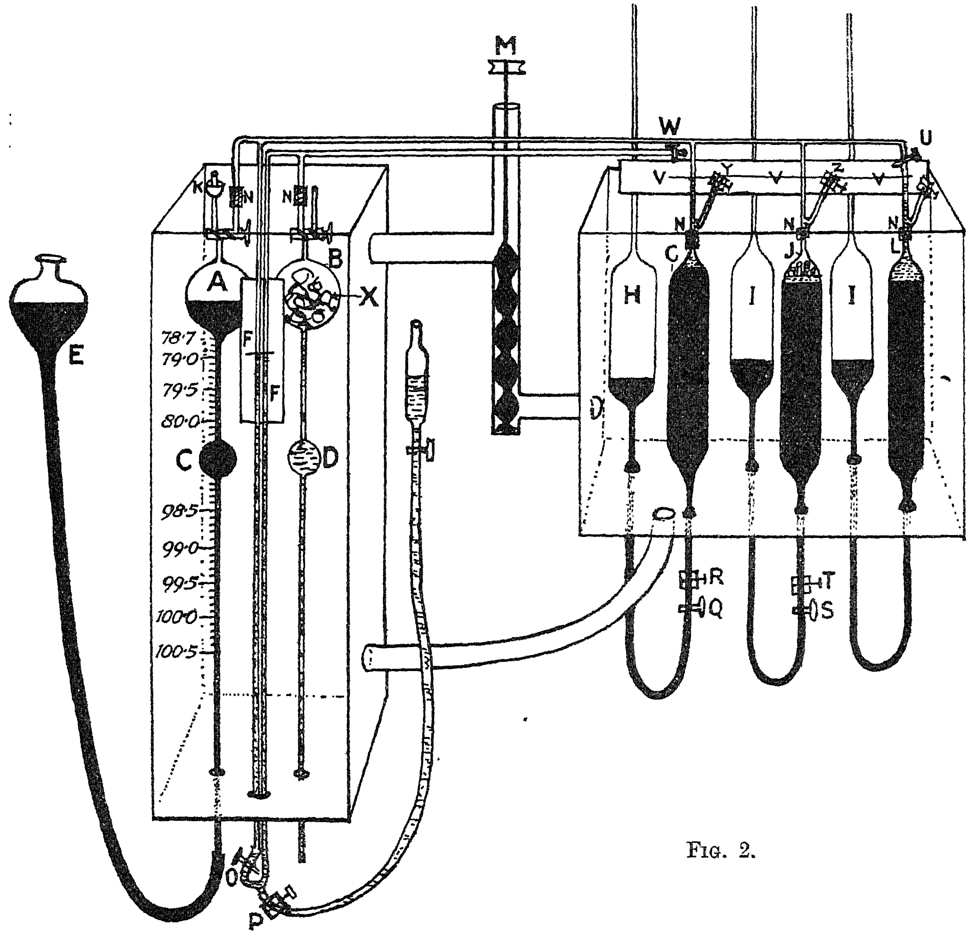


Fig. 2.

It consisted of a 100-c.c. burette A, with two bulbs A and C, and a carefully graduated stem, as on sketch. An exactly similar one served as the control burette B, and both were placed in a large copper water-bath. Adjustment of pressure was effected by tubes from each burette passing in front of a mirror F, which was marked with a line ; the tubes contained dilute acid up to the mark and a fine adjustment was made by screw clip P. A tap or clip was inserted at O, which was turned off completely except when reading results.

The control burette B had fine absorbent cotton strands *x* within it, dipping into 5 per cent. sulphuric acid. After trials it was found more accurate to have the lower bulb D and the tube up to bottom of bulb B filled with the acid. It was fitted with a side-tube with a tap W, this had the same volume as the tubes coming from the burette A.

The pipettes for absorption were contained in a separate large copper water-bath with glass front, which was connected to the first bath by wide copper tubes, as in sketch. A spiral mixer M, driven by a small motor, kept the water rapidly circulating through the two baths. Pipette G contained 0·3 c.c. of 5 per cent. sulphuric acid over the mercury, and was used for adjusting the vapour tension of the gas after it had been over the pyro :

Pipette J contained 2 c.c. of 10 per cent. potash for absorption of CO_2.

Pipette L contained 10 c.c. of 10 per cent. pyrogallic acid in 1·55 specific gravity potash ; it was fitted with a tap U above the pipette to obviate any possible absorption of oxygen during the CO_2 determination.

The pipettes G and J were fitted with side-tubes Y and Z for easy renewal of reagent, and they had taps Q and S below with screw clips R and T on the rubber tube just above, for the fine adjustment of the height of the liquid to a line on a mirror V.

There were rubber connections between pipettes and tubes at N, and the lower ends of these tubes were made funnel-shaped to avoid air being caught as small bubbles. To make this funnel end, seal the tube and blow a bulb on the end, which is cut in half as in fig. 3.

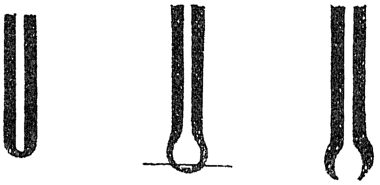


Fig. 3.

The pipettes were filled with mercury and each had a reservoir which could be raised or lowered, the stem passing through a short length of rubber tube attached to a collar on the bottom of the water-bath. The pressure of the mercury was not allowed to exceed 2 inches. The apparatus was proved to be air-tight, gas left in it not changing appreciably in volume over two or three days.

Before use the apparatus is filled with nitrogen by doing a blank experiment. The burette is then filled with the sample, the mercury being allowed to over-run into the cup K, fig. 2, so that no air is introduced in the tubing between the burette proper and the sampler. The volume is adjusted by means of control burette (previously in communication, for a moment, with outside air, by tap W) and tubes in front of F, the tap O being open and P used to adjust. O is then turned off and the gas passed over into pipette J for absorption of CO_2; after half a minute it is returned to A; again passed over to J; the process being repeated four times; the gas is then approximately adjusted and allowed to stand several minutes; it is then finally adjusted and must remain constant for a few minutes as a check upon the validity of the reading. After the measurement, the fluid in F is brought up to the junction with connecting tube to eliminate dead space and the gas passed 10 times into L, then twice into J, it is then passed once into G, each time waiting half a minute; after returning to burette A it is adjusted and measured as before.

A sample test with laboratory air gave 79·098 N, 20·862 O_2, 0·040 CO_2, the generally accepted average figures for the outside atmospheric air being 79·04, 20·93 and 0·03 respectively.

Different samples of the same gas mixture from a sealed chamber gave the following consecutive results: (1) 0·523, (2) 0·522, (3) 0·525, (4) 0·525, and the same gas 4 days in the sampler before analysis gave: (5) 0·527, (6) 0·520 per cent. of CO_2.

Calculation of Results.

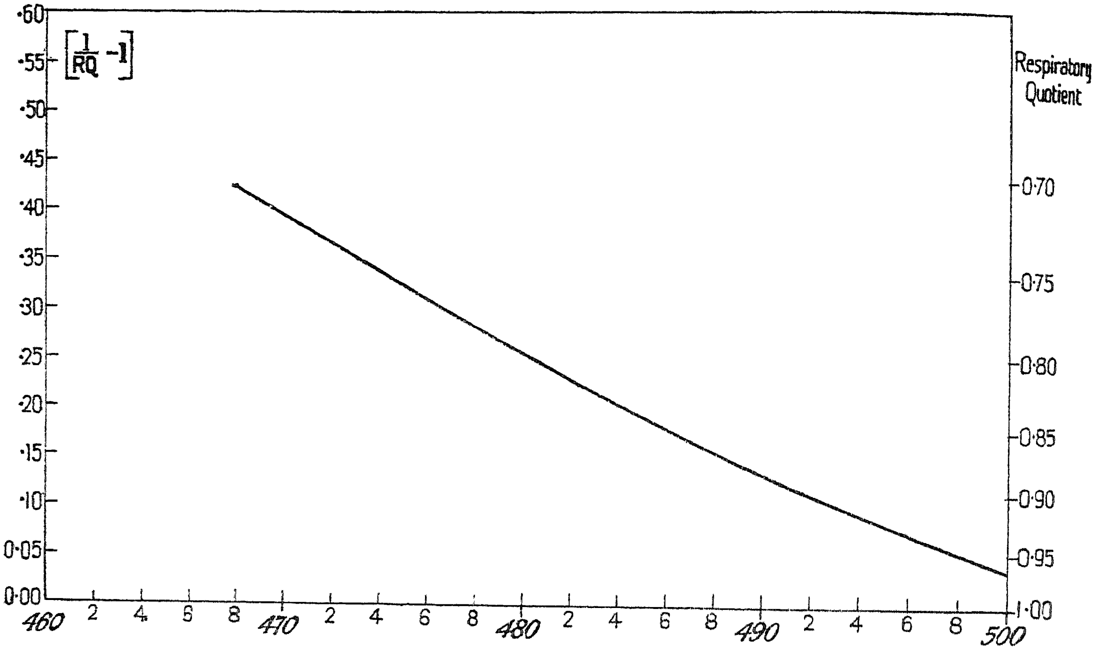
The amount of CO_2 excreted when multiplied by a factor dependent upon the respiratory quotient gives the number of calories produced by the subject; this is divided by the surface area of the subject determined by the Du Bois charts and gives the heat loss per square meter of body surface; which according to Rubner's law is a reliable index of body metabolism.

We have also in our calculation used figures based upon the empirical equations as given in tables by Harris-Benedict (R), which take into account not only the height and weight but the age of the subject, and in general are claimed by the authors to give results deviating from the mean by much less than the weight-height charts only.

The respiratory quotient is given by the "carbon dioxide excreted" divided by the "oxygen absorbed," *i.e.*, the oxygen originally present less the oxygen found. For our purpose a chart (fig. 4) was constructed to minimise calculations, giving the calorie equivalent of 1 litre carbon dioxide from $\frac{1}{RQ - 1}$ which is equal to $\frac{1{\cdot}265 \text{ nitrogen increase}}{CO_2 \text{ excreted}}$. Later we obtained the respiratory quotient by Kroghs' method, which is simpler and quicker (see reference later).

It will be seen that the RQ can be obtained from the same chart by inspection.

Having obtained the percentage of CO_2 in the chamber before and after experiment, the difference multiplied by the chamber capacity, 10,050 litres, gives the litres CO_2 formed during the experiment. The volume obtained is



Factor for calories per litre CO_2.

FIG. 4.—Calorie value chart.

subject to the following corrections: (1) Temperature correction to 0° C.; (2) barometric correction to 760 mm.; (3) Water vapour pressure correction to 0° C. 760 mm. pressure; (4) correction for slight known continuous leak of chamber. This latter correction is rarely necessary, and can be avoided by suitably adjusting the temperature of the chamber before and during experiment.

(1), (2) and (3) have been combined together in a chart (fig. 5) on the basis of a 100 per cent. saturation with water (which is usually the case as shown by the hair hygrometer).

When the saturation is not 100 per cent. the results can be calculated from the usual published tables for (1) and (2) together, and the vapour pressure separately.

The corrected volume of CO_2 excreted is multiplied by the factor obtained for the respiratory quotient giving the number of calories; this figure is converted into calories per hour and calories per 24 hours; the former is divided by the surface area from the following chart fig. 6 to give heat loss per square

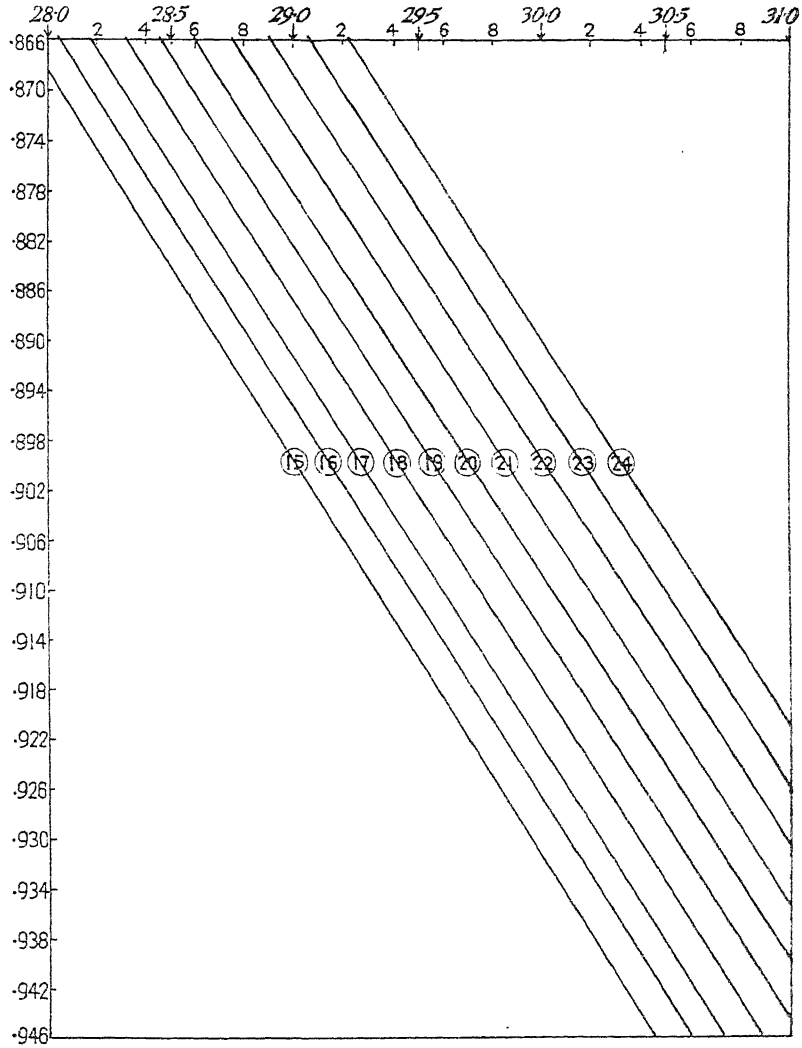


FIG. 5.—Chart for factor to 0° C., 760 mm., dry gas.

metre, the normal figure being 40; and the latter when subtracted from the standard metabolic rate figure for the patient, according to Harris-Benedict table, gives the difference in calories from the standard on the basis of 24 hours.

Conditions of Experiment.

The subject had a diet of low protein on the day previously, meat being excluded. Nothing was eaten after the usual light supper at 8 p.m., and the fasting subject next morning at 8 a.m. lay upon a bed or sat in a chair in the

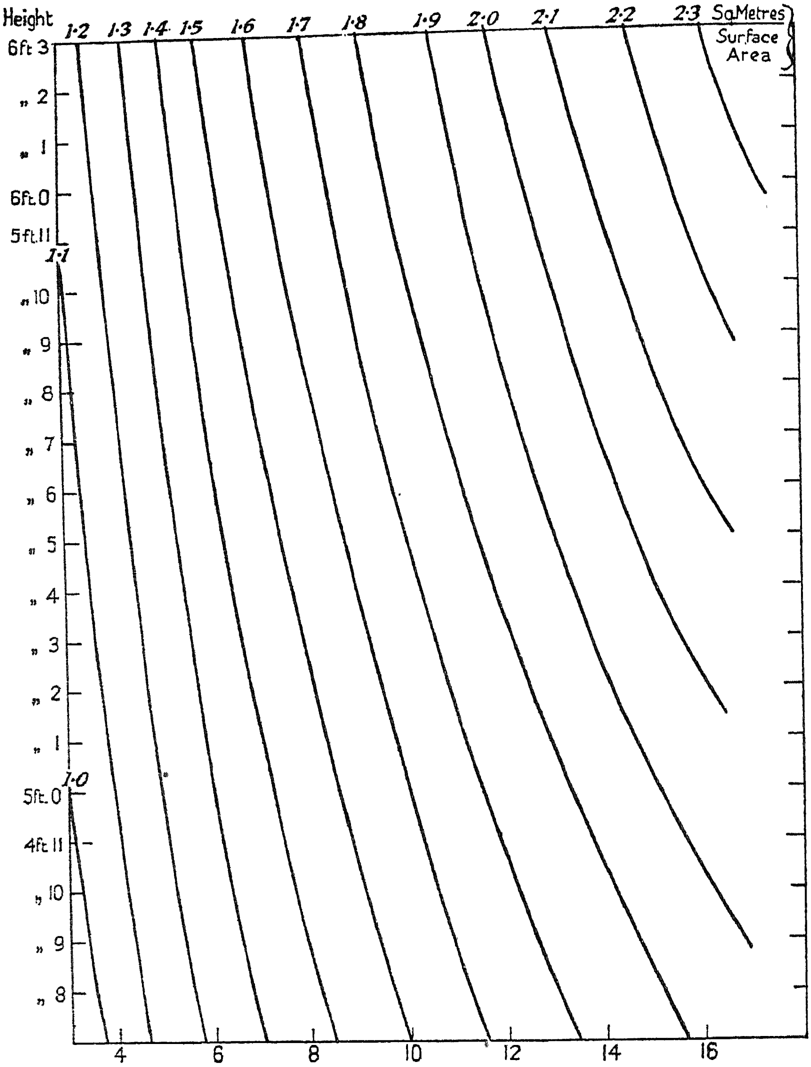


Fig. 6.—Surface-area Chart. (Adapted from Dubois, 'Archiv. Int. Med.,' vol. 17, 1917.)

Note.—The accuracy of 1 per cent. absolute for CO_2 excreted was easy to obtain, but a similar accuracy for the oxygen absorbed could not be relied upon, since the differences were extremely small. The determination of oxygen has, therefore, now been discontinued, and the respiratory quotient and calorie value obtained by dieting the subject, who is not allowed meat the day before the test. This gives a standard respiratory quotient of 0·86 which has a calorie factor of 4·86. The procedure is fully described in a paper by Krogh (R). We satisfied outselves that this figure was approximately correct in 11 consecutive cases examined, before definitely adopting this procedure.

chamber for a period of half-an-hour or more. The chamber door was then closed, the fan switched on full and, after 5 minutes at least, the first sample was taken. A record was then made of the time, pressure indicators, temperature, and humidity of the chamber, and the barometric pressure outside the chamber also was noted.

In 2 hours the second sample was taken and the CO_2 determined in the two samples. Calculations then gave the basal metabolic rate of the patient.

The temperature of the chamber was maintained usually between 15° C. and 18° C., and was not allowed to change much during an individual experiment. The fan was kept revolving at a moderate speed during the whole of the period, and was switched on full for not less than 5 minutes previous to taking each sample. In several cases the blood sugar at the end of the experiment was determined.

The results of the experiments are given in Part II of this paper.

Thanks are due to the late Sir Frederick Mott for suggesting and supervising this work, to Prof. J. S. Haldane and Mr. Ivon Graham of the Mining Research Laboratory, Birmingham University, for valuable suggestions and assistance in the devising and construction of the apparatus ; also to the Medical Research Council for the cost, approximately £100, of the chamber.

The work was done in the Laboratories of the Joint Board of Research for Mental Diseases, City and University of Birmingham.

Summary.

A new method for the determination of the basal metabolic rate is described. The advantages claimed are the diminution of such emotional disturbances as fear, anxiety and excitement, with consequent muscular relaxation in the subject. This enables figures for the basal metabolic rate to be obtained which are much lower than those given by the bag methods. It is submitted that figures so obtained approximate more closely to the real basal metabolic rate.

This is accomplished by the use of a specially constructed air-tight chamber in which the subject lies or sits for a period of 1 or 2 hours. The change in the composition of atmosphere is measured by a modified Haldane air-analysis apparatus, and the modifications necessary in order to obtain the somewhat greater accuracy required are fully described, and tables given for calculating results.

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PART II.

The determination of the basal or standard metabolic rate of subjects has occupied the attention of a very large number of workers both in this country and in America, and the extensive literature on the subject testifies to the enormous amount of work done in this direction.

The new method described in Part I of this paper gave figures for metabolic rates, obtained from normal subjects, which were very much below those figures given by other workers using the bag method, and to investigate this discrepancy a considerable number of experiments on normal subjects had to be carried out. Examination of the tables appended shows that the results obtained with normal controls, although in many cases in close agreement with one another, show certain wide differences under slightly varying conditions; these differences are not to be attributed to the apparatus, which gives results accurate to about 2 per cent., whilst the difference in a normal subject under varying conditions of previous rest, extreme motionless attitude, and trained voluntary muscular relaxation, may amount to 20 per cent. or more. The various factors concerned in maintaining or increasing the "basal" metabolic rate are not measurable as separate entities, but are associated and may influence one another. Some of these factors may be eliminated however, at least in part giving results which must therefore be regarded as approaching the true basal rate.

Energy Components of Metabolism.

The various energy transformations occurring in normal metabolism can be grouped as follows:—

Table I.

A.—*Muscular Movements*	(1) Voluntary. (2) Semi-conscious and reflex.
B.—*Muscular tonus*	(1) Postural. (2) Due to attention.
C.—*Involuntary muscular movements*	(1) Heart action and respiration. (2) Peristalsis.
D.—*Metabolism in the production of internal and external secretions and nervous activity.*	

Of these the theoretical basal energy is represented by C and D, when these are at the minimum of physiological activity. In order to obtain figures which allow of the estimation of the correct relative values of C and D in different subjects, it is necessary that the other factors should be standardised as nearly as possible. Such standardisation is very difficult when the factors A and B are large; and it is obvious that in practice inhibition of muscular tone in a case of hyperthyroidism is much more difficult than in a myxœdematous subject. Nevertheless it is very desirable that these factors should be eliminated as far as possible, and this is obtained by placing the subject under conditions of rest and comfort, such as are possible in the respiration chamber described in Part I of this paper.

A and B are by no means small when a face-piece or mask is used, as in the bag and spirometer methods, especially if the valve or the tube resistance is at all appreciable; also attention complicates the muscular tonus: factors which vary considerably and are not capable of accurate standardisation. B (2) is a factor which must be taken into consideration even in the chamber method, especially when the subject is in for the first time.

D is minimal after a lapse of some hours following a meal when anabolic and catabolic processes have attained some kind of equilibrium; it is also largely dependent upon the presence in the blood of certain endocrine secretions. Emotional states, with the concomitant liberation of internal secretions and the effects therefrom, also must have a very profound effect upon B (2), C and D. Sleep theoretically eliminates nearly all the variable undesirable factors, and it is upon metabolism during sleep that we hope to follow up the present research.

Metabolism of Normal Subjects under varying conditions.

It is stated that the basal metabolism of normal subjects may vary 10 per cent.; 15 per cent. must be considered abnormal pending proof to the contrary (*c*).

The following table demonstrates the effect upon the reduction of metabolism of voluntary mental and bodily relaxation. This is not easy at first, owing to slight degrees of normal fear processes, involuntary attention, and interest in the strange surroundings; but by practice a progressive ability to inhibit muscular tone and mental effort is shown to be paralleled by a reduction in metabolism.

Table II.—Voluntary Relaxation of Mind and Body.

Subject quite quiet, being instructed to relax muscles and make no voluntary mental effort.

Subject.	CO_2 output per minute c.c. actual.	CO_2 output per minute reduced to standard.*	Metabolism percentage.†
2	223	230	95·7
	215	221	92·0
	210	216	90·0
	187	193	80·3
	185	192	80·0
	177	184	76·8
3	217	231	96·4
	180	192	80·0
	182	194	81·0
	175	187	78·8
	167	178	73·6
4	233	224	93·6
	198	190	79·5
	183	176	73·6
	181	174	72·5
5	213	226	94·6
	200	213	88·6
	193	205	88·8
	187	199	82·7
	187	199	82·7
	180	191	79·8

* Figures corrected for deviation from the average height, weight, age and sex, assuming a normal heat production of 1,680 calories per 24 hours (MacLeod) and proportional calculation on the basis of the Harris and Benedict tables (Ref. *b*).

† Figures obtained by dividing the actual metabolism in calories for 24 hours by the theoretical calories for the same individual as given in the Harris and Benedict tables and multiplied by 100.

It will be seen by inspection that the ability of the subject to effect relaxation of mind and body is paralleled by a corresponding reduction in metabolic rate.

The above figures are given in the order of their determination, but are not necessarily consecutive.

A fully-trained subject is able to inhibit muscular tone, voluntary movements and mental processes so completely that the metabolism is reduced by 20 per cent. or more below the normal; under these conditions the body state approaches that of sleep, in fact sometimes it is difficult not to go to sleep. This reduction of metabolism is often followed or accompanied by a sense of cold and occasional fatigue.

Effect of Extreme Relaxation of Mind and Body.

Table III.

Subject and experiment.	CO_2 output actual per minute.	CO_2 output per minute reduced to standard.	Metabolism percentage.
1D	162	196	81·5
1E	169	204	85·0
2P	187	193	80·3
2R	185	192	80·0
2U	177	184	76·8
3G	180	192	80·0
3I	182	194	81·0
3K	175	187	78·8
3M	182	194	80·8
5H	175	186	77·4
5I	187	199	82·7
8A	164	169	70·5
8B	176	181	75·7
8C	177	182	76·4
8D	173	178	74·4
8H	184	190	79·2
8I	168	173	72·3

17 experiments. Average CO_2 per minute = 188. Average metabolism 78·4 per cent.
5 normal subjects.

Note.—In experiment 1E shivering movements, due to cold, were noted. In experiment 8A and 8I the subject afterwards complained of fatigued condition.

It will be seen that normal subjects, trained to relax mind and body, have a metabolism which is 20 per cent. or more below the standard metabolism for the same subject as determined by the bag methods.

Metabolism of Normal Subjects with varying conditions.

Table IV.

Standard conditions of diet, etc., as described in Part I, except where otherwise stated.

(1) *Extreme relaxation of mind and body.*

Average of 17 experiments on five normal subjects.

CO_2 output per minute (reduced to standard) = 188 c.c. per minute.

Metabolism 78·4 per cent. of the standard.

(2) *Reading easy book.*

No special relaxation ; subject quite at ease lying on bed.

Subject and experiment.	CO_2 output per minute actual.	CO_2 output per minute reduced to standard.	Metabolism percentage.
4C	233	224	93·6
11A	228	243	101·1
13A	232	232	96·5
14A	212	210	87·3
15A	219	272	113·5
16A	198	235	97·9
17A	177	220	91·6
18A	161	191	79·8
19A	174	215	82·3
20A	172	204	84·7

Average of 10 experiments, 225 c.c. ; average metabolism 92·8 per cent.

In many of the above experiments the subject was in the chamber for the first time.

(3) *Partial attention to surroundings.*

Subject instructed to relax mind and body as completely as possible ; quiet ; no voluntary movement whatever.

—	Subject.	CO_2 output actual.	CO_2 output reduced to standard.	Metabolism percentage.
First time in chamber	5	213	226	94·6
Second time in chamber....	5	200	213	88·6
Repeat with another subject—				
First time in chamber	57	184	223	93·3
Second time in chamber	57	172	209	87·3

Where subject is in the chamber for the first time a higher figure for metabolism is noted, which becomes less as the strangeness of the surroundings ceases to attract attention.

(4) *Normal diet.* Full breakfast at 8 a.m.

Time.	CO_2 output actual.	CO_2 output reduced to standard.	Metabolism percentage.
10–11	217	231	96·0
11–12	210	223	93·0
Control fasting—			
2 days previous	193 } 190	205 } 203	85·8 } 84
1 day after . . .	187	199	82·7

(A difference of 14 per cent. increase 2–3 hours after a meal and 10 per cent. increase 3–4 hours after a meal over that of a fasting condition. The control was obtained by taking the mean of the preceding experiments and the next subsequent experiment on special diet.)

Note.—King states "a breakfast of 220 calories increases the basal metabolism by 7 per cent. for the first hour only, 2 per cent. for each of the two hours following."

(5) *Feeling of irritation.*

——	CO_2 output per minute actual.	CO_2 output reduced to standard.	Difference.	Metabolism.
(*a*) Wrong book taken into chamber to read	240	264	69	110·4
Compare previous experiment in preceding hour when subject did not not know of the error.—Mental quietude	177	195		81·2
(*b*) Painful stiff neck	233	242	58	100
Compare lowest metabolism at mental quietude for same subject	177	184		76·8

(6) *Reclining in armchair compared with lying on bed.*

——	CO_2 output per minute actual.	CO_2 output reduced to standard.		Metabolism.	
Reclining in armchair	183	176		73·6	
Lying on bed, hour immediately previous to above....	198	190	182	79·5	76·0
Lying on bed, hour immediately succeeding	181	174		72·5	

Subject slept a short time on each occasion he was on the bed. A decrease of 3·2 per cent. in armchair as compared with lying on bed.

Note.—This is in agreement with King's findings of 3 per cent. See also Delcourt Bernard and André Mayer (*d*).

(7) *Hot Bath. Subject Fasting and on Special Diet.*

——	CO_2 output actual per minute.	CO_2 output reduced to standard.	Metabolism.
Hot bath 10.30 a.m.—			
Experiment, 11 to 12	191	197	82·0
,, 12 to 1	175	180	75·1
Hot bath, 10.10—			
Experiment, 10.30 to 11.30	174	186	77·5
,, 11.30 to 12.30	146	156	64·9

The comfort consequent upon a hot bath enables the subject to relax his muscles more easily, and the state of mind approaches that of sleep. In addition there is not the demand for the usual production of body heat.

(8) *Mental Effort.*

Experiment.	CO_2 output actual per minute.	CO_2 output reduced to standard.	Difference.	Metabolism.
(A) Reclining in armchair imagining the process of violin playing, *i.e.*, mentally rehearsing concert pieces....	210	224	32	93·3
Control experiment-mental quietude	180	192		80·0
(B) Repeat of above— Mental effort	190	203	9	84·5
Mental quietude	182	194		81·0
(C) Counting vowels in book, paying great attention to accuracy	262	252	28	105·2
Control experiment with identical movements	233	224		93·6
(D) Mentally preparing a lecture	215	237	0	98·8
Control	215	237		98·8
(E) Repetition with same subject. Reversing order of determination— Mental quietude	209	230	29	96·1
Mental effort	235	259		108·3

Control experiments were done in the preceding or succeeding hour, with such movements as unavoidably occurred in the experiment repeated as nearly as possible. Further work is needed in this direction, since highly variable factors are involved which are difficult to standardise or allow for ; in general it appears that mental effort increases metabolism directly and also through the increased muscular tonus.

(9) *Effect of Sleep.*

		Experiment.	CO_2 output actual per minute.	CO_2 output reduced to standard.	Metabolism percentage.
Subject 3.	X	Asleep to within 5 minutes of end of experiment	150	158	65·7
	Z	Asleep, opened eyes once	142	149	62·4
	AA	Turned over whilst asleep	174	183	76·2
	AD	Soundly asleep	142	149	62·3
	AE	Asleep all the time	141	148	61·8
	AF	Asleep all the time	134	142	59·2
	AG	Moved head	178	188	78·8
	AH	No movement	163	173	72·0
	AI	Very slight movement of head once	164	174	72·5
Subject 4.	H	Moved head-shoulder once slightly	178	174	72·7
	I	Moved slightly once	190	186	77·5
	J	Experiment started within 3 minutes of commencing sleep	170	166	69·4
	K	Sound asleep	170	166	69·4
		Average	– –	166	70·0

Additional experiments connected with Sleep.

Subject 3.	A	Subject on full diet. Dinner at 7.15 p.m., experiment at 2.30 a.m. Soundly asleep	185	195	81·1
		Ditto, experiment 3.30 a.m. Soundly asleep	167	176	73·2
	B	Awake and alert just after asleep continuing 20 minutes into experiment. Subject remained perfectly motionless	201	212	91·5

The experiments started immediately sleep supervened, as shown by an electrical recording instrument which demonstrated a sudden loss of tone in muscles of the hand. Another experiment, in which subject turned over from right side to left whilst still sleeping, had a total metabolism of 76·5 per cent. in the hour.

(10) *Fatigue.*

Experiment.	CO_2 output actual per minute.	CO_2 output reduced to standard.	Metabolism.
Walked 3 miles, rested ¾ hour before experiment	192	205	81·3
,, 10 ,, ,, 1 hour	165	174	72·3
,, 10 ,, ,, 2 hours	182	191	79·7
,, several miles, rested 1½ hours	178	174	72·7

Muscular relaxation is favoured by previous excessive muscular effort.

(11) *Playing Violin.*

	Experiment.	CO_2 output actual per minute.	CO_2 output reduced to standard.	Metabolism.
A	Easy pieces from memory	315	336	140
	Difficult exercises—Perpetuum Mobile, Ries; Andante Concerto, Elgar	437	466	192·7
B	Easy pieces from memory	400	427	178·4
	Difficult pieces—Kreutzer Sonata, Max Bruch Concerto	484	516	215

The player adjusted the actual movements to correspond as closely as possible in each pair of experiments. Attention during violin playing increased the metabolism by about 30 per cent. over that for playing easy pieces.

(12) *A Case of Myxœdema.*

This was a case of thyroiditis treated by X-ray; resulting in destruction of all thyroid substance; as shown by subsequent histological total absence of thyroid epithelium, and chemical total absence of iodine in the fibrous remnant.

CO_2 output per minute actual .. .. .. 102 c.c.
CO_2 reduced to standard .. .. .. 133 c.c. per minute.
Metabolism percentage .. .. 55·4.

This gradually increased by thyroid feeding in a few months to 70·7 per cent., and upon cessation of the thyroid treatment it fell again in a few months to nearly the original low figure, *i.e.*, 56·6 per cent.

A protocol of an actual experiment is given :—

Name.—D.L.W.
Date.—May 14, 1926.
Time of last meal.—8 p.m., May 13.
Any meat yesterday.—No.
Age.—25.
Height.—5 feet 9 inches.
Area.—1·72 (1)
Weight.—9 stone 7 lb.
Calories H and B.—1,596 (2)

Experiment.—		*Start.*	*Finish.*
	Time	2.25 a.m.	3.25 a.m.
	Indicator A	42·1	42·3
	,, *B*	23·4	23·8 (3)
	,, *C*	10·0	9·9
	Humidity	90	93
	Temperature	16·7° C.	16·8° C.
	Bar. Pressure	29·40	
	Factor to N.T.P.	0·905	(4)
(5)	CO_2 *(per cent.)*	0·175 } 0·179 } 0·177	0·291 } 0·293 } 0·292
Calculations.—			
	CO_2 *excreted*	0·115	
	CO_2 *per hour*	0·115	
	Litres, CO_2 *excreted*	11·55	
	,, *at N.T.P.*	10·45	
	CO_2 *per minute c.c.*	174	
(6)	,, *reduced to standard*	183	
(7)	*Calories equivalent, per hour*	50·8	
	,, *per day*	1,220	
Basal metabolism.—			
(8)	*Heat loss square metre*	29·5	
	Calories difference, H and B	−376	
(9)	*Metabolism per cent.*	70·5	

Notes.—Asleep all the time, but turned from right side to left, apparently without waking, also moved left leg slightly once, no other movement. This experiment followed immediately on a preceding experiment 1.25 to 2.25 a.m.

(1) From table, Part I of this paper.
(2) From tables, reference (*b*).
(3) Indicates leakage 10 litres from chamber during experiment = 0·1 per cent.
(4) See table, Part I.
(5) At least two gas analyses are made on each specimen of gas withdrawn from chamber, figures usually agree to within 0·003 c.c. per cent.
(6) See footnote, p. 176.
(7) Factor litres CO_2 to calories 4·86.
(8) Divide heat production per hour by surface area.
(9) See footnote, p. 176.

The details of the above metabolic rate determinations are to be found in the Annual Reports of the Joint Board of Research for Mental Diseases (*e*).

No reliable statement can as yet be made with regard to the real basal metabolic energy balance-sheet, but certain combinations of the energy factors are shown in Table IV. The depression of metabolism in relaxation of the mind and body would be accompanied by a proportionate decrease in the general needs of the body, and therefore of the metabolism of heart and respiration. The difference between first and second times in the chamber is probably due to slight degrees of fear and attention increasing the muscular tonus.

The effect of food in the one experiment carried out is greater than that usually recorded. Irritative stimuli cause a profound disturbance in metabolism, as might be expected. Mental effort is to be correlated with a diffuse muscular tonus increase.

A hot bath, by adding to the comfort of the subject and also diminishing the need for production of body heat, induces a condition of muscular relaxation with a considerable reduction of metabolism. The effect of sleep needs further correlation with the depth of sleep. It is well known that the depth of sleep in the first hour is very great as compared with the later hours; it is interesting to note an increase in metabolism in the period following sleep, which must be correlated with the sudden reappearance of muscular tonus and possible release of internal secretion. In this connection it has been shown that rapid awakening causes biochemical changes in the body identical with those accompanying fear processes. The effect of playing the violin with and without mental effort shows that there is a large increase in metabolism due to the increased muscular tonus accompanying concentration of attention. Fatigue causes a lowering of metabolism.

It is thought that many of the above experiments are complicated by a certain amount of muscular tonus due to attention; especially as in several experiments the subject was in the chamber for the first time, and it is hoped to show, by further experiments carried out whilst the subject is sleeping, that the real basal metabolism is even lower than the figures given.

Summary.

The numerous determinations of the basal metabolic rates by the bag method have involved certain variable and preventable factors, such as muscular tonus and attention, which may have considerably obscured the results; and the usual accepted limits by these methods of up to 15 per cent. are too large, so that more refined methods of investigation are needed.

The present work shows how figures can be obtained which approach more nearly the true basal rate; and by reducing the magnitude of certain variable factors results more than 20 per cent. lower than those by the bag method are obtained with normal subjects.

The effect of various factors upon the metabolism has been studied, and figures illustrating the effect of relaxation of mind and body, sleep, fatigue, diet, irritation, hot baths, etc., are given.

My thanks are due to the late Sir Frederick Mott for suggesting this work; to Prof. J. S. Haldane and Mr. Ivon Graham for valuable help, and to Dr. T. C.

Graves, Superintendent of the Hospital, for encouraging interest and for help in the clinical arrangements. We are also indebted to the Medical Research Council for defraying the cost of the respiration chamber.

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(*d*) Delcourt Bernard and André Mayer. 'Comptes Rendus Soc. de Biologie,' vol. 92, No. 5, 1925.

(*e*) 'Annual Reports of the Joint Board of Research for Mental Diseases, City and University of Birmingham, 1925 and 1926.'

On the Effect of the Ovarian Hormone in Producing Pro-Œstrous Development in the Dog and Rabbit.

By S. A. Asdell, M.A., Ph.D., and F. H. A. Marshall, Sc.D., F.R.S., Institute of Animal Nutrition, Cambridge.

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The question of the existence and identity of the hormones secreted by the follicular part of the ovary is one which has of recent years attracted much attention. Allen and Doisy (1923) recently opened the discussion by showing that the follicullar fluid of the sow and of other animals contains a substance which, when injected into the spayed rat, causes the development of the type of vaginal epithelium found in this and other species during œstrus, *i.e.*, the cornified type, several cells thick, in place of the basement cell layer with a single thickness of cubical cells. They also found that the processes induced by the injections into the spayed rat are usually followed by a definite œstrus during which coitus can occur. Frank (1923), however, has remarked that in the rodent coitus is a poor criterion of the sexual condition of the female. This is a fair criticism, for rodents in general show great catholicity in their time of acceptance of the female, while the rat in particular shows a decided preference for coitus in the dark, when observation is difficult, though in this species the finding of the vaginal plug with spermatozoa is convincing proof that coitus has taken place. Since the publication of Allen and Doisy's work, the effect of liquor folliculi injections has been confirmed by Dickens, Dodds and Wright (1925), who, in common with most other workers, have accepted the condition of the vaginal smear as their criterion of œstrus. Very recently Parkes and Bellerby (1926) have shown in them ouse that the œstrus-producing effect is not confined to follicular extract, but is quite as pronounced if extract of ovarian " stroma " is employed.

External signs of "heat" are but scanty in the rat and the mouse, and do not afford an ideal means of determining the activity of an extract. It has been thought desirable, therefore, to test the effects of follicular extracts on animals which show more definite external changes, more particularly in the pro-œstrum. Thus the bitch and the rabbit have been employed and the results are recorded in this paper. They suggest that the external signs of pro-œstrum and œstrus may not be caused by precisely the same agency as that which is responsible for the internal development at these stages of the cycle. Several subsidiary points have arisen during the progress of this work, but as their investigation is not completed, they will be left for a further communication.

As recorded by Marshall and Halnan (1917), the bitch experiences usually two periods of œstrus in the year at an interval of about six months. After the prolonged anœstrum there is a period of pro-œstrum, or building up, followed by a certain amount of extravasation of blood from the uterine mucosa and external bleeding from the vulva lasting about a week. This is followed by the period of œstrus, also a week, when the bitch will accept the male. This is usually succeeded, if fertile coitus does not occur, by pseudo-pregnancy lasting up to eight weeks, after which rapid involution of the reproductive organs (and more particularly the uterus) sets in and a long anœstrum supervenes.

The bitch exhibits several advantages for this work, for coitus is readily performed under observation and the vulva swells during pro-œstrum and remains visibly enlarged and congested during œstrus. The period of extravasation of blood in the uterus is shown by a flow from the vulva during pro-œstrum. In addition, advantage may be taken of the anœstrous period to observe whether the injections have any effect on the ovaries as well as on the other reproductive tissues.

The rabbit usually ovulates only after coitus and accordingly experiences under laboratory conditions prolonged periods of œstrus, during which the vulva is congested. This species also readily accepts the male during œstrus, there being no doubt when coitus has occurred, but copulations may take place during pregnancy and pseudo-pregnancy.

The liquor folliculi for injection was derived chiefly from pigs' ovaries obtained from several sources. The liquor was aspirated from the follicles and placed in twice its volume of 95 per cent. alcohol. After standing for a week or more, the precipitated proteins were filtered off and the alcoholic washings were added to the filtrate. The filtrate was evaporated under reduced pressure, the residue dissolved in ether, filtered, and the ether evaporated off when the material was required for injection. Injections of the material suspended in Ringer's solution were made subcutaneously in the flank.

The Bitch.

The bitches were all small smooth-haired terriers. Where possible, the injections were made at a definite time in the anœstrous period and at 24-hour intervals. Towards the end of the period of injection, which was usually 10 days, the site of injection tended to become slightly swollen.

Before killing the bitches, each was tested with a male dog in order to decide whether the psychic reactions necessary for coitus were occurring. In no case did a bitch show the slightest sexual interest in the dog, which made repeated attempts at serving her. The vulva was also carefully examined, but no swelling was observed in any of the bitches. In one bitch daily measurements were made. These showed no variation. Vaginal smears were not taken, as it was decided that it would be better to rely on the appearance of sections of the vaginal wall for evidence of hormonic action. Insertion of a swab through the vulval opening might conceivably have caused a swelling of the margins by irritation.

The quantity of fluid injected, together with the conditions of the experiment and the observed results, are given in Table I. In all cases the injections were made at a time when bitches are not usually on heat

It is clear that as a result of the injections the simple cubical vaginal epithelium with its single additional layer of flat basement cells has developed into a stratified layer such as is known to occur in the guinea-pig and rat at the time of pro-œstrum and œstrus, and also in the latter species as a result of the injection of extract of liquor folliculi. This stratified epithelium was found to break up under the influence of prolonged injections. The cells in most of the specimens were losing their organisation and becoming cornified. Heavy injections of corpus luteum extract produced a slight development of the vaginal epithelium, but the effect was not pronounced and may have been due to inclusions of liquor folliculi, for the corpora lutea used were of very recent formation.

Table I.

Bitch.	Interval between subsidence of vulval swelling and beginning of injections.	Treatment.	Condition of uterus.	Condition of vagina.
I	11½ weeks	No injections	No congestion; glands present, ill developed.	—
II	11½ weeks	No injections	No congestion; glands more developed than last, but not greatly. Fluid in lumen of uterus.	—
III	(No œstrus observed)	No injections	No congestion; glands ill developed.	Basement epithelium and one layer of cubical cells.
IV	10 weeks; injections continued till 11½ weeks, when killed.	Daily injections for 10 days each injection = 1 cystic, 3 normal ovaries. Liquor folliculi extract.	Marked congestion; development of glands. (Corpora lutea visible in ovaries.)	Stratified epithelium, cornification occurring in outer layers.
V	(No œstrus observed)	Daily injections for 5 days, each injection = 12 pairs ovaries. Liquor folliculi extract.	Some congestion; fair development of glands, fluid in lumen of uterus.	Cornified flattened cells, layer intact.
VI	12 weeks; injections continued till 13½ weeks, when killed.	Daily injections for 10 days, each injection = 12 pairs ovaries. Liquor folliculi extract.	Marked congestion; some development of glands.	Cornified flattened cells, layer breaking down.
VII	(No œstrus observed)	Daily injections for 10 days, each injection = 12 pairs ovaries. Liquor folliculi extract.	Marked congestion; some development of glands, fluid in lumen of uterus.	Cornified flattened cells, layer breaking down, but not so extensively as last.
VIII	12½ weeks; injections continued till 14 weeks, when killed.	Daily injections for 10 days, each injection = 3 pairs ovaries. Corpus luteum extract.	Marked congestion; very marked development of glands, which approach pseudo-pregnant state.	Two layers of somewhat flattened cells in addition to the cubical and basement cells.

Table II.

Rabbit.	Condition.	Treatment.	Condition of uterus.	Condition of vagina.
I	Mature, spayed	No injection	Columnal epithelium, very little mucosa, few glands, not vascular.	Columnal epithelium, not vascular, surface in simple folds.
II	Mature, spayed	7 days injections = 5 pairs ovaries each day. Liquor folliculi.	Cubical epithelium, mucosa moderately developed, glands fairly plentiful, moderately vascular.	Columnar cells longer than in I; more vascular, surface in complex folds.
III	Mature, on heat	No injections, killed immediately after coitus.	Cubical epithelium glands and mucosa well developed, marked congestion.	Tall columnar cells vascular, surface in simple folds.
IV	Mature, on heat	No injections, killed immediately after coitus.	Cubical epithelium mucosa and glands well developed, marked congestion.	Tall columnar cells, vascular, surface in complex folds.
V	Immature, spayed	No injections	Very small, considerable mucosa in proportion to muscle, non-vascular, very few glands, cubical epithelium.	Single layer of cubical cells, not vascular, surface in simple folds.
VI	Immature	7 days injections = 5 pairs ovaries each day. Liquor folliculi.	Cubical epithelium, glands well developed, marked congestion.	Small, but larger than V, single layer columnar epithelium, outer *edge of cells breaking up.*

The uterine mucosa under follicular injections became congested, there was a marked increase both in the number and in the size of the vessels, and a definite glandular development. The uterine effect appeared to be the more pronounced where injections had been made over a longer period.

The Rabbit.

In these experiments two sets of rabbits were used, one set being mature and the other immature. Both sets were spayed and daily injections of follicular extract were made for seven days after 14 days had elapsed from the time of the removal of the ovaries. At the close of the period of injections the rabbits were tested with the buck, but refused to copulate. They were then killed and the reproductive organs were examined. Details are given in Table II.

The Rat.

An attempt to obtain the hormone in a state of purity is being made in co-operation with Mr. H. E. Tunnicliffe. In the course of this work, in which the vaginal smear method controlled by microscopic examination of sections of the vaginal wall of the rat is being used, the occurrence of the stratified type of epithelium is confirmed. The uterus is far less useful as an indication of hormonic activity, for this again in the spayed rat undergoes fairly rapid atrophic changes and much larger doses of follicular extract must be employed to produce congestion and glandular growth. This is in accord with the experience of Dickens, Dodds and Wright (1925).

Discussion.

In the first place it must be stated that the injection of liquor folliculi extract had no effect on the follicular system of the ovary of the anœstrous bitch. Milk was secreted by bitch IV (see Table I) on the second day of the injections, and, on killing, fairly large corpora lutea were found in the ovary. This bitch is therefore omitted from further consideration, for a sufficiently long time may not have elapsed since the closing phase of the pseudo-pregnant period. In subsequent injections this difficulty was allowed for by waiting for a longer time before starting the injections.

It was not possible to employ many bitches in this work; nevertheless, the histological results were so clear as to be quite convincing. It is evident that the injection of the extract in the bitch and in the rat causes a growth of the resting vaginal epithelium, two cells thick, into a stratified epithelium ten or more cells thick. This is well illustrated by the sections from the vagina of bitch V in which the injections were made for five days only. In the bitches VI and VII, in which the injections were continued for a total of ten days, cornification was well advanced and breakdown of the epithelial layer had set in.

The effects produced on the uterus are of special interest, for injection of follicular extract produced marked congestion together with a growth of the glands of the mucosa; in fact, the changes were practically identical with those which take place in the bitch during the early stages of pro-œstrum (Marshall and Jolly, 1905). The degree of development does not approach that of pseudo-pregnancy, which process is under the control of the corpus luteum (Marshall and Halnan). It must be pointed out that on the assumption that the œstrous growth of the uterus and of the vaginal epithelium is due to a single substance, the uterus-stimulating hormone of the corpus luteum must be different, for it has no effect on the vagina. The injections of corpus luteum extract in bitch VIII suggest that the uterine conditions of pseudo-pregnancy may be reproduced, but similar injections which have been made into spayed rabbits were without effect.

There is no certain correlation between the duration of the injection period and the effect on the uterus, as the latter is influenced by the degree of retrogression which has to be made up.

Neither the bitches nor the rabbits would copulate after the injections. The vulvæ of the bitches did not show the usual swelling of the pro-œstrum, although the internal changes were very pronounced. This vulval swelling may be caused by some other factor, or it may take place later than the other changes described, though in the latter case one would have expected some slight swelling. The vulvæ of the rabbits showed none of the congestion which is usually seen at œstrus. In this species, too, although marked uterine changes were brought about, no stratified epithelium was formed in the vagina. The columnar epithelium appeared to have increased slightly by addition to the length of the individual cell. Control rabbits which were on heat, being killed one minute after coitus, showed an exceptionally high type of columnar epithelium in the uterine end of the vagina. This type of cell with the nucleus of its proximal end is described by Tsu (1924) as typical of "post-œstrus," œstrus itself being characterised by the occurrence of stratified epithelium consisting of polyhedral cells. The latter are described as being pushed out by the growth of columnar cells below, and after lacunæ containing cell débris and degenerating leucocytes have developed, this layer is said to be desquamated, to be succeeded by ciliated epithelium which, rapidly losing the cilia, becomes columnar epithelium of the high type. The columnar cells described here certainly occurred during œstrus, for the rabbits copulated, large follicles were present, and the vulvæ were congested. They had probably been on heat for some time, as they had not recently bred.

Summary.

(1) Injections of follicular extract obtained from solution in alcohol produce typical pro-œstrous changes in the uterus of the bitch during the anœstrous period (that is, at a time when the uterus is normally quiescent).

(2) Injections of the extract likewise cause a growth of the vaginal epithelium (which becomes stratified) followed by a breaking down of the cornified cells.

(3) Changes can be brought about similarly in the uterus and vagina of the rabbit, but in the latter animal no stratified epithelium was formed in the vagina.

(4) In the experiments described no definite œstrus was experienced by either the bitches or the rabbits and there was no swelling of the vulva ; it is possible, therefore, that in the actual production of œstrus a further factor may be involved.

(5) The injections had no apparent effect on the follicular system of the ovary.

The experimental work was carried out at the Field Laboratories, Cambridge. The injections were done by S. A. Asdell.

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The Measurement of Percentage Hæmolysis.—II.

By Eric Ponder.

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(From the Department of Physiology, Edinburgh University.)

In previous papers (1, 2, 3) we have shown that the reaction between a simple hæmolysin, such as saponin or one of the bile salts or soaps, can be described by an expression not unlike that for a monomolecular reaction, provided that it is recognised that this reaction takes place among a population of cells each member of which possesses a different resistance to the lysin, the resistances being distributed according to a frequency curve.

In the papers quoted it has been shown that such a conception of the reaction accounts in every way for the time-dilution curves for the simple lysins, and for the phenomena observed when the concentration of cell suspension is varied; the percentage hæmolysis curves alone remain to be discussed in full, although in (1) it has been demonstrated that the principal properties of these curves may be explained on the above assumptions.

In this paper we propose to deal with the percentage hæmolysis curves for different lysins, in relation to the theory put forward in (1), and to extend the theory to cover cases in which the lysin acts in the presence of inhibitors and accelerators.

I.—Methods.

For the finding of the percentage hæmolysis curves we employ two methods, each of which will be described in some detail.

Radiometer Method.

In principle this method has been described in a previous paper (2), and some of the results obtained recorded. Certain disadvantages, however, attach themselves to the use of the apparatus already described; these are overcome in the model constructed as below, the general appearance of which may be seen from an inspection of fig. 1.

The light is supplied by a 200-watt Ediswan projection lamp, which is contained in a metal cylinder, and which can be made to slide towards the radiometer on two parallel rails. This form of lamp provides a very concentrated and intense beam.

The radiometer is enclosed in a cylindrical water-jacket, the lower part of which is prolonged forwards so that a small chamber, about 1 inch deep and of

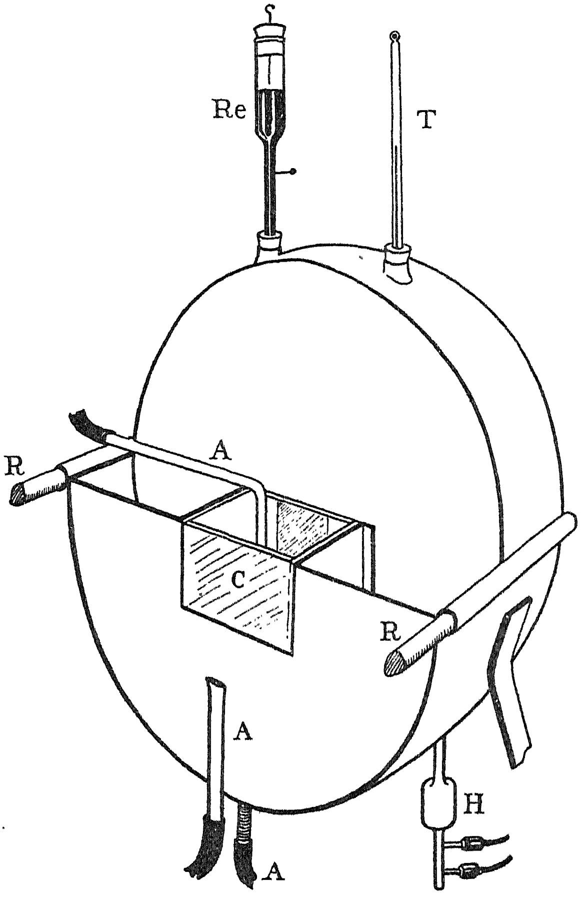

Fig. 1.—Water-jacket of radiometer. RR, rails on which lamp slides; AAA, air blasts; H, heating unit; Re, thermo-regulator; C, glass cell containing suspension; T, thermometer.

20 c.c. capacity, lies between the light and the vanes of the radiometer. The two walls of this chamber which are in the path of the light are of glass; the other walls are of metal. The construction is such that four of the five walls are in contact with the water of the water-jacket, while the top of the chamber is open. Into the chamber passes a fine tube carrying compressed air for the purpose of mixing the cells. Into the water-jacket project an electrical heating unit, an automatic temperature regulator, usually set to 25° C., a thermometer, and a tube carrying compressed air for the purpose of stirring. A

lens is fixed into the back of the water-jacket so that the movements of the vanes of the radiometer can be observed.

The radiometer is of the four-vane type, and selected for its sensitiveness, in respect to which different instruments differ greatly. It must be carefully levelled by the levelling screws on which the whole apparatus stands.

In order to prevent heating from the lamp, a strong blast of air is arranged to pass upwards in front of the lamp, between it and the glass wall of the chamber in which the cells are placed. A water-jacket, containing circulating cold water, may be placed in the same position.

The instrument must be calibrated before each experiment, the calibration being carried out much as in the case of the earlier model, but with obvious modifications to meet the altered construction. In an actual experiment the lysin together with all other components of the hæmolytic system except the cells is placed in the chamber and allowed to assume the temperature of the water -jacket. The volume of the fluid introduced initially is 16 c.c. At the same time the cell suspension is placed in an adjacent water-bath and warmed to the same temperature. When all are warmed, 4 c.c. of the suspension is added to the chamber with a warmed pipette, and the readings at once commenced. Readings are taken at suitable intervals until the completion of lysis, and are afterwards converted into values for percentage hæmolysis by means of the calibration curve.

The advantages gained by this particular method are the following :—(*a*) Owing to the strong light it is possible to work with suspensions of the same strength as we usually employ, instead of with suspensions of a quarter the strength, as was necessary in the older apparatus. Further, the light source is variable, and any strength of suspension within limits can be employed. (*b*) Under the more intense light the movements of the radiometer are much more brisk and associated with less lag. (*c*) The chamber being small, comparatively little material is needed—20 c.c. instead of 100 c.c. (*d*) The necessity of regulating only one water-jacket and the fact that this is kept constant in temperature by an automatic regulator is a great simplification and convenience.

Selenium Cell Method.

In this method the change in conductivity of selenium when exposed to light is used to measure the amount of light passing through the suspension, and thus indirectly the amount of percentage hæmolysis. The method was, so far as we know, first applied to hæmolysis by Mellanby ; to make it a method of precision, however, certain details must be carefully observed, and so we shall describe our modification in detail.

A selenium cell is connected to one arm of a bridge, the other arm having a resistance box attached; an accumulator (2 or 4 volt), a short-circuiting key, and a moving coil mirror-galvanometer complete the circuit. The selenium cell is held on a rack and pinion, so that it can be moved to and from the light source. The cell should be hooded to protect it from stray light.

The light source is provided by a small projection lamp lit from a 4-volt accumulator by one of two circuits. In the one circuit is a Morse tapping key, pressure of which lights the lamp. In the other circuit is a clock which, every 30 seconds, lights the lamp for a period of 5 seconds by the completion of the circuit. The lamp is enclosed in a small chamber from which the light emerges to pass through the glass face of a water-jacket within which is the cell containing the hæmolysing suspension. Having traversed the water-jacket and the glass cell it falls on the selenium.

The suspension and the other components of the hæmolytic system are contained in a small glass cell of optical glass, in section about 1 cm. by 1·3 cm.; when 4 c.c. of fluid are in the cell the light has to traverse the fluid before reaching the selenium. The suspension is kept stirred by a fine jet of air, and the water-jacket is heated by a small heating unit. The general lay-out of the apparatus is shown in fig. 2.

The apparatus is calibrated in the following way. A series of standards are prepared to represent percentage lysis from 0 per cent. to 100 per cent. by placing in each of 11 bottles 5 c.c. of distilled water. To the first bottle nothing is added, to the second 0·01 c.c. of blood is added from the finger, to the third 0·02 c.c. of blood, to the fourth 0·03 c.c., and so on until the eleventh bottle, which receives 0·1 c.c. of blood. The cells are allowed to hæmolyse, and 5 c.c. of 1·7 per cent. NaCl is then added to each bottle. Again blood is added from the finger: 0·1 c.c. to the first bottle, 0·09 c.c. to the second, and so on until the eleventh bottle is reached, which receives nothing. The contents of the bottles then represent 0 per cent., 10 per cent., 20 per cent. . . . 100 per cent. hæmolysis. The blood used in making up the standards is, of course, derived from the same source as the blood used in making the cell suspension to be used in the particular experiment.

Each standard is in succession placed in the glass cell, and the deflection of the galvanometer which corresponds to that particular degree of lysis observed. This is done in the following way. When the glass cell containing the suspension is in position, the key completing the circuit containing the clock is closed;

each 30 seconds the light is switched on automatically by the clock, and the galvanometer deflects. The maximum of the swing is noted in each case, the galvanometer being thus used as a ballistic instrument. The seventh reading is taken as the correct one.

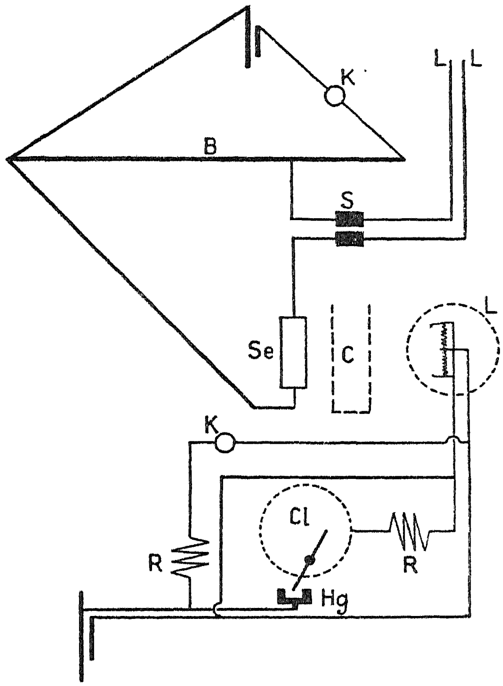

FIG. 2.—L, lamp; Se, selenium; C, suspension cell; B, bridge; KK, keys; RR, 4-ohm resistances, variable; LL, leads to galvanometer; S, short-circuiting key; Cl, clock; Hg, mercury trough.

The reason for this procedure is that it is difficult to get the galvanometer to record a constant deflection when the selenium is exposed to light for any long period, for the spot of light usually settles in a deflected position for a short time and then begins to creep towards the galvanometer zero. This tendency for the deflection to lessen is very marked if the light is turned on the whole time, and, moreover, the first deflections tend to be greater, for the same light intensity, than subsequent ones. If, however, the instrument is used as a ballistic instrument, and if the exposures to the light are of short duration, the deflections soon become constant, and so we take the seventh reading as the correct one, thus allowing for the tendency of the deflections to lessen. The readings are taken at 30-second intervals so as to make the values obtained during calibration as nearly alike to those obtained in an actual experiment as possible.

In an actual experiment the lysin and all other components of the system except the cells, are placed in the glass cell; the volume of fluid used is 3·2 c.c. The glass cell is placed in the water-jacket and allowed to assume its temperature. The warmed cell suspension, 0·8 c.c. in amount, is kept in a thermos flask, and added at a suitable moment; readings of the galvanometer deflection are taken as they automatically occur every 30 seconds. These readings are afterwards converted into values for percentage hæmolysis by means of the calibration curve. If readings are required at intervals other than 30 seconds, the circuit containing the clock can be switched off, and the other lighting circuit used.

As regards utility, the two methods are much alike. The advantages of each may be seen by inspection of the two calibration curves (fig. 3). It will be observed that the radiometer is more sensitive in the region of 0 per cent. to 50 per cent., and the selenium cell more sensitive as complete lysis approaches. The initial parts of the curves are thus best investigated with the radiometer, while the final parts are best explored with the selenium cell. In this way the two methods can be used to check each other.

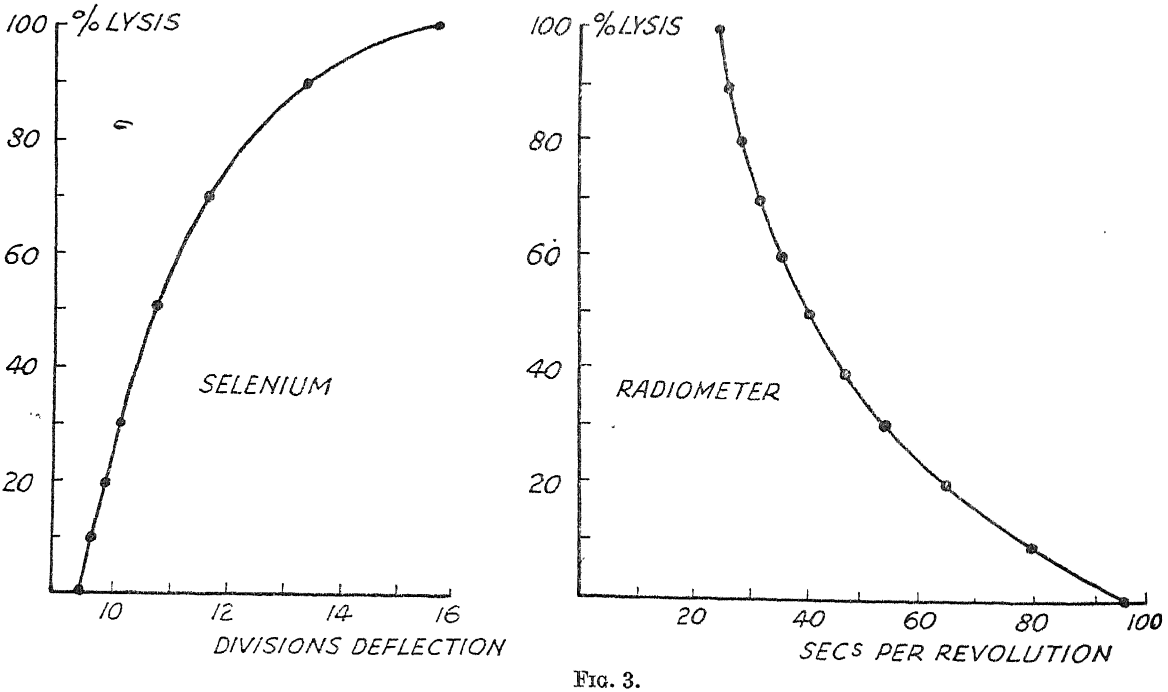

Fig. 3.

All other methods referred to in this paper will be found described in earlier papers, particularly in (2), (3) and (4). The standard blood suspension referred to is the same as that used in earlier recorded experiments, and consists of the thrice washed cells from 1 c.c. of blood, finally suspended in 20 c.c. of 0·85 per cent. NaCl.

II.—CELL-LYSIN SYSTEMS.

In (1) we have shown that for a suspension of a constant arbitrary surface the expression

$$\kappa t = \log \frac{c}{c - x} \qquad (1)$$

describes the course of the fundamental reaction between the lysin and the particular cell component affected by it. In this expression t is the time, c the initial concentration of lysin, x the amount of lysin transformed in combination with the cell component, and κ a constant. This fundamental reaction must be imagined to proceed in a population of cells of various resistances, the resistances being measured in terms of x, the amount of lysin transformed before the cell will undergo lysis, and the resistances being distributed according to some form of frequency curve. We have now (*a*) to find the exact form of frequency curve which will represent the distribution of the resistances, and (*b*) to show that, if this resistance distribution be postulated, the form of the percentage hæmolysis curves for the lysin acting in different concentrations is exactly accounted for. The procedure is best shown by recording actual experiments.

1. *Saponin.*

In this experiment we study the time-dilution and percentage hæmolysis curves for the system in which saponin acts on a standard suspension of erythrocytes. Proceeding in the usual way, the following values for points on the time-dilution curve at 25° C. were obtained :—

Dilution, 1 in	c, mgs.	t, exper.	t, calc.
10,000	0·20	0·85	0·94
20,000	0·10	1·8	2·0
30,000	0·06	3·7	3·6
40,000	0·05	5·5	5·5
50,000	0·04	8·6	8·7
60,000	0·03	19·0	19·1

This curve is shown in fig. 4, and is described by expression (1) in which $x = 0{\cdot}032$ and $\kappa = 0{\cdot}185$. The degree of correspondence may be seen from the calculated values of the above table, or from the closeness with which the experimental points lie on the calculated curve in the figure.

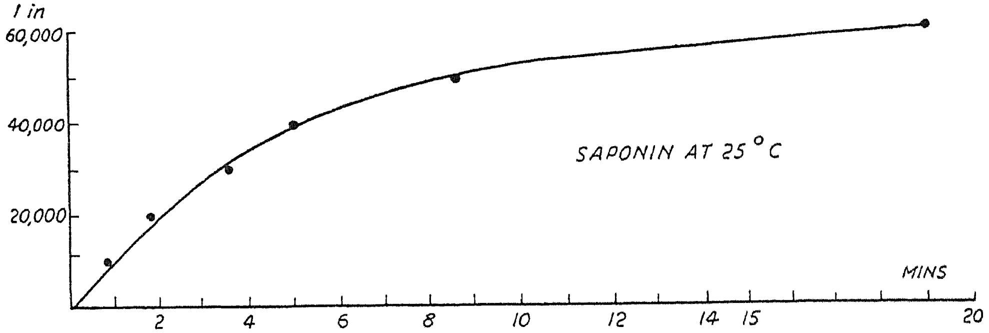

Fig. 4.

Selecting a convenient dilution of lysin, 1 in 50,000, we now find the curve for percentage hæmolysis by means of the radiometer and the selenium cell. This curve, at 25° C., is as follows :—

Time.	Percentage lysis.	Time.	Percentage lysis.
0	0	4·5	68
0·5	0	5·0	80
1·0	0	5·5	88
1·5	2	6·0	93
2·0	5	6·5	96
2·5	11	7·0	97
3·0	20	8·0	99
3·5	34	8·7	100
4·0	54		

This curve, shown in fig. 5, is sigmoid, presents a distinct latent period, and has obviously a negative skewness.

Making t and x variables in expression (1), we can calculate the amount of lysin used up from moment to moment during the reaction when $c = 0{\cdot}04$ and $\kappa = 0{\cdot}185$. Such calculation gives the following values :—

Lysin used up, x in mgs.	Time.	Lysin used up, x in mgs.	Time.
0·0025	0·3	0·0200	3·7
0·0050	0·7	0·0225	4·4
0·0075	1·05	0·0250	5·3
0·0100	1·55	0·0275	6·2
0·0125	2·0	0·0300	7·5
0·0150	2·5	0·0320	8·7
0·0175	3·0		

Since we have the amount of lysin used up from moment to moment during the course of the reaction, and also the number of cells hæmolysed from moment to moment, we can draw up a table showing the relation of the amount of lysin transformed to the number of cells hæmolysed. As the amount of lysin transformed when a cell hæmolyses is our measure of the resistance of that cell, we can thus construct from the data the curve giving the distribution of the resistances of the various members of the population. From the last table and the curve for percentage hæmolysis the following table can be drawn up :—

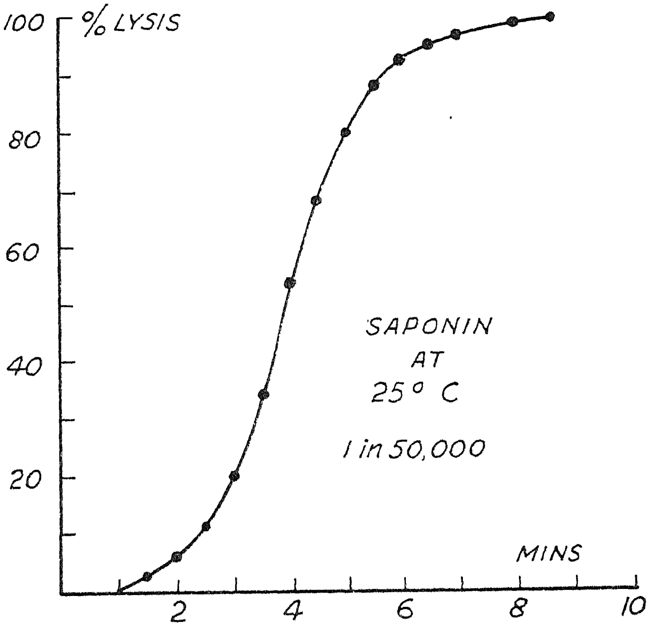

FIG. 5.

Lysin used up, x in mgs.	Percentage number of cells.	Lysin used up, x in mgs.	Percentage number of cells.
0·0025	0	0·0200	20
0·0050	0	0·0225	26
0·0075	0	0·0250	19
0·0100	2	0·0275	9
0·0125	3	0·0300	4
0·0150	6	0·0320	2
0·0175	9		

Plotting the number of cells against the group corresponding to the lysin transformed, we obtain a frequency polygon as in fig. 6. This polygon shows the resistance distribution of the various cells of the population to the lysin, the resistance being measured in terms of the amount of lysin which requires to react with the cell component affected in order to produce lysis.

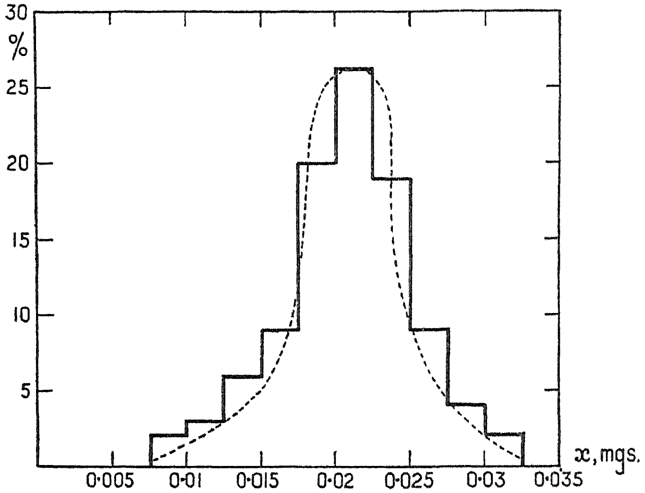

Fig. 6.

To this polygon a frequency curve may be fitted in the usual way. Calculation of the moments and of the criterion gives

$$\mu_1 = 3\cdot27 \qquad \beta_1 = 0\cdot1143$$

$$\mu_2 = -2\cdot02 \qquad \beta_2 = -9\cdot851$$

$$\mu_3 = -104\cdot4 \qquad \kappa = 0\cdot0013.$$

From these values it appears that Type II of Pearson's curves will supply a satisfactory fit, with the values

$$m = -2\cdot646$$

$$a = -5\cdot01$$

$$y_0 = 26\cdot2$$

inserted in the equation for the curve,

$$y = y_0\left(1 - \frac{x}{a}\right)^m. \tag{2}$$

The curve which results from the use of this expression and these values is drawn through the polygon, and provides a sufficiently good fit for our purpose. In previous papers we have always assumed that the distribution was of the type

$$y = y_0 \cdot e^{-b^2x^2}, \tag{3}$$

but though it appears that a curve of Type II fits the data better than one of Type VII, the theory already advanced is subject to no essential alteration.

It has now to be shown that the reaction described by expression (1), with the values $x = 0 \cdot 032$ and $\kappa = 0 \cdot 185$ inserted, taking place in a population with resistances distributed according to expression (2) with the values of the constants as above, is sufficient to account for the form of the percentage hæmolysis curves for saponin in all dilutions, and not only in a dilution of 1 in 50,000.

Taking the dilution 1 in 40,000, we obtain the percentage hæmolysis curve given below. Besides the experimental results are placed the results as calcuated from expressions (1) and (2).

Time.	Percentage lysis, experimental.	Percentage lysis, calculated.
0	0	0
0·5	0	0
1·0	2	1
1·5	7	5
2·0	12	11
2·5	28	26
3·0	55	53
3·5	75	75
4·0	87	87
4·5	95	95
5·0	98	99
5·5	100	100

The agreement is remarkably good, and shows the extent to which the theory can be applied. As usual, the greatest errors are in the region of 0 per cent. hæmolysis.

Next taking the dilution 1 in 60,000, we proceed in the same way. The experimental and the calculated results are compared in the following table :—

Time.	Percentage lysis, experimental.	Percentage lysis, calculated.
0	0	0
1	0	0
2	0	2
3	6	8
4	18	20
5	37	40
6	60	63
7	75	77
8	86	86
10	95	95
15	98	99
19	100	100

Again the correspondence is very good, considering the difficulties of work with such a dilute lysin.

One of the most interesting points shown in all these curves is the presence of a distinct latent period during which the reaction goes on without the lysis of any cells. This period appears because no cell of resistance less than 0·0075 milligrammes of lysin is found in the resistance distribution, and until the amount of lysin is transformed no cell will hæmolyse.

In a previous paper we have suggested as a rough measure of the " skewness " of the percentage hæmolysis curves the value of the ratio t_{100}/t_{50}, t_{100} being the time taken to hæmolyse the whole population, and t_{50} being the time required for the lysis of 50 per cent. of the cells. As has been pointed out in the paper quoted (1), the ratio t_{100}/t_{50} is equal to

$$\frac{\log \dfrac{c}{c - x_{100}}}{\log \dfrac{c}{c - x_{50}}} \tag{4}$$

where x_{100} is the amount of lysin transformed in the lysis of the whole population, and where x_{50} is the amount of lysin transformed in the lysis of the first 50 per cent. of the cells, and where the latent period is assumed to be negligible. This expression gives quite good results in a number of instances, but if the latent period be of appreciable length it requires some modification, since then x_{50} is not equal to half of x_{100}, as no cells are hæmolysed during the latent period. The exact relation is

$$x_{50} = \frac{x_{100} - x_l}{2} + x_l \tag{5}$$

in which x_l denotes the amount of lysin transformed during the latent period. Now if t_l be the length of the latent period, we have the relation

$$\frac{t_{100} - t}{t_{50} - t_l} = \frac{\log \dfrac{c - x_l}{c - x_{100}}}{\log \dfrac{c - x_l}{c - x_{50}}} \tag{6}$$

in place of expression (4), where x_{50} has the value as given by expression (5). This provides a rough measure of the skewness, just as does expression (4) in the cases where the latent period can be ignored. In the case of the curves given above, the skewness, when measured in this way, works out at about 2·8.

It may be mentioned that this method of dealing with the skewness is absolutely dependent on the symmetry of the frequency distribution. This fact requires careful note, for it sometimes happens that expression (4) appears applicable to the data, but that nevertheless there is a distinct latent period. Under such circumstances it may be safely assumed that the resistance distribution is not symmetrical, and that it would be better described by a curve of Type IV than by one of Type II. Some such cases are mentioned in (1). Cases also occur where the latent period is so short as to be negligible, and to these cases expression (4) seems to apply, providing that the distribution of resistances is a symmetrical one.

We have considered this particular way of expressing the "skewness" of the percentage hæmolysis curve because it forms a very rapid and convenient way of determining whether or not the resistance distribution is symmetrical. All the values required in expression (5) can be obtained easily from the time-dilution and percentage hæmolysis curves; the fulfilment of the equality indicates at once that the resistance distribution is symmetrical without the necessity of working out the exact form of the frequency curve or even the form of the frequency polygon.

2. *Sodium Taurocholate.*

In the case of lysis by sodium taurocholate there is reason to believe that the hæmoglobin liberated by the less resistant cells as they hæmolyse acts as an accelerator of the reaction (*see* 1), and that the acceleration of the reaction is brought about by a change in the form of the resistance distribution. To obtain the resistance distribution to this lysin we proceed, however, in a similar way to the above.

At 25° C. we obtain a time-dilution curve for sodium taurocholate acting on the standard suspension, and find that this curve is described by expression (1) in which $x = 0\cdot38$ and $\kappa = 0\cdot25$. Selecting a dilution of lysin of 1 in 4,000 the following percentage hæmolysis curve is obtained at the same temperature.

Time.	Percentage lysis.	Time.	Percentage lysis.
0	0	3·0	40
0·5	0	3·5	62
1·0	5	4·0	80
1·5	11	4·5	90
2·0	19	5·0	95
2·5	30	5·7	100

These figures give the frequency polygon :—

Lysin used up, x, in mgs.	Percentage number of cells.	Lysin used up, x, in mgs.	Percentage number of cells.
0·05	0	0·25	18
0·10	3	0·30	33
0·15	7	0·35	24
0·20	9	0·38	6

This polygon is shown in fig. 7. It is obviously not symmetrical, for the median lies nearer to the upper extreme than to the lower extreme. Instead

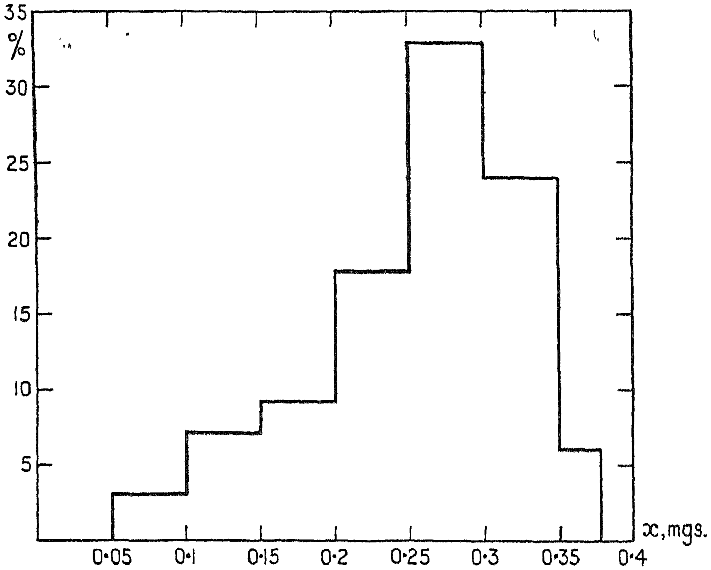

Fig. 7.

of being at $x = 0\cdot21'5$, it lies at $x = 0\cdot28$. According to the assumptions made in (1), the median should lie at $0\cdot7075x_{100}$, after we allow for the fact that the acceleration of the fundamental reaction by the liberated hæmoglobin cannot commence until the end of the latent period. In this case we have the median, or x_{50}, lying at $0\cdot6969x_{100}$, a value exceedingly close to that which we should expect.

Here we have to draw attention to a fact observed since the publication of the first of these studies (2); this close agreement does not always occur. In several cases we have found the median to lie at a position from $0\cdot60x_{100}$ to $0\cdot65x_{100}$, and in a few cases analysis has shown the frequency distribution to be symmetrical, and the percentage hæmolysis curves to be as in the case of saponin, where the outpoured hæmoglobin has no accelerating effect on the reaction. These cases are the exception rather than the rule, but they occur nevertheless, and attention should be drawn to the fact, for the methods used are such that they cannot be attributed to experimental error. Why these exceptions should occur we cannot at present say.

In order to show that the frequency polygon obtained above is a correct one, we may apply it to the results obtained with another dilution of lysin, 1 in 5,000. Using the same constants for the fundamental reaction as above, and the same frequency polygon as obtained from the percentage hæmolysis curve of 1 in 4,000 lysin, we obtain the calculated percentage hæmolysis curve given below. Beside the calculated figures are placed the experimental ones. The curves of this and the experiment with the lysin dilution of 1 in 4,000 are also shown in fig. 8, the experimental points being marked on the calculated curve.

Time.	Percentage lysis.		Time.	Percentage lysis.	
	Calc.	Exper.		Calc.	Exper.
0	0	0	5	60	63
1	2	0	6	76	75
2	11	10	7	85	83
3	22	20	10	99	98
4	38	40	12	100	100

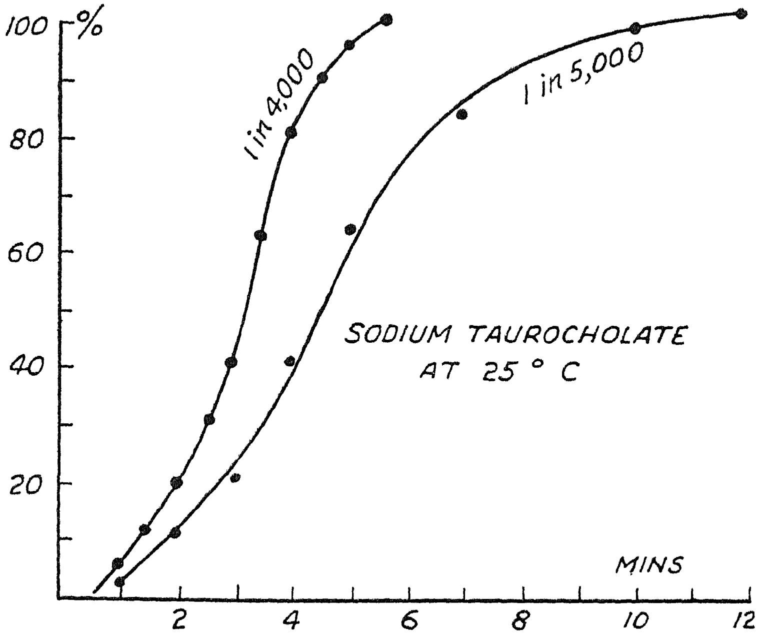

FIG. 8.

This correspondence between calculated and observed values is quite satisfactory.

Since this paper is principally concerned with technique and with fundamental results, we do not propose to deal with the percentage hæmolysis curves which are given by such lysins as ammonia, sodium hydrate, or the soaps. The first two lysins give curves resembling those for saponin, and capable of analysis by the same methods. The curves for the soaps are very difficult to obtain with accuracy, owing to the opacity which occurs with these lysins. With the exception of sodium and potassium oleate, which give curves of the same type as those given by sodium taurocholate, most of the soaps resemble saponin in their lytic properties. The frequency distribution of the cell resistances to all of these lysins may be found by the same methods as used in the above examples.

III.—CELL-LYSIN-ACCELERATOR SYSTEMS.

We have now to find the form of the percentage hæmolysis curves when the system contains an accelerator. Two important cases arise: (*a*) when the accelerator is present from the commencement of the reaction, and (*b*) when the accelerator is added during the reaction.

(*a*) In determining the form of the curves when the accelerator is present initially, a special modification of the technique is necessary, whereby the cells are allowed to remain in contact with the accelerator before the lysin is added. If this is not done, and if lysin and accelerator are mixed and the cells added to the mixture as they would be added to a solution of simple lysin, a curious distortion of the initial part of the percentage hæmolysis curve results, presumably because, as soon as the cells are added, both lysin and accelerator commence to react with the cell components simultaneously. If the cells are allowed to remain in contact with the accelerator for a period before the lysin is added, the reaction between accelerator and cell component is complete at the moment of addition of the lysin. Undistorted percentage hæmolysis curves are then obtained.

We therefore add to 1·6 c.c. of a suitable concentration of the accelerator to be used, 0·8 c.c. of the standard cell suspension, both fluids being warmed to 25° C. before mixing. The mixture, contained in the small glass cell which fits into the water-jacket of the selenium apparatus, is allowed to remain at this temperature for a measured time, usually about 5 minutes; the warmed lysin, 1·6 c.c. in amount, is then added, and the readings at once commenced. For most experiments with these systems, readings have to be made at 10 or

15-second intervals, instead of at 30-second intervals, as lysis is very rapid indeed.

1. *Saponin and Aspartic Acid.*

Selecting a dilution of 1 in 40,000 saponin, and analysing the percentage hæmolysis curve by the above methods, we find the following curve to represent the integral of the frequency distribution of the cell resistances to the lysin itself:—

Lysin used up, x in mgs.	Percentage lysis.	Lysin used up, x in mgs.	Percentage lysis.
0·005	0	0·025	65
0·010	9	0·030	89
0·015	20	0·035	100
0·020	35		

This curve was obtained from the percentage hæmolysis curve, and from the values $x = 0·035$, $\kappa = 0·238$, these being found from the time-dilution curve at 25° C., at which temperature the entire experiment was carried out. The curve appears in fig. 9.

The percentage hæmolysis curve for the system containing 1 in 40,000 lysin,

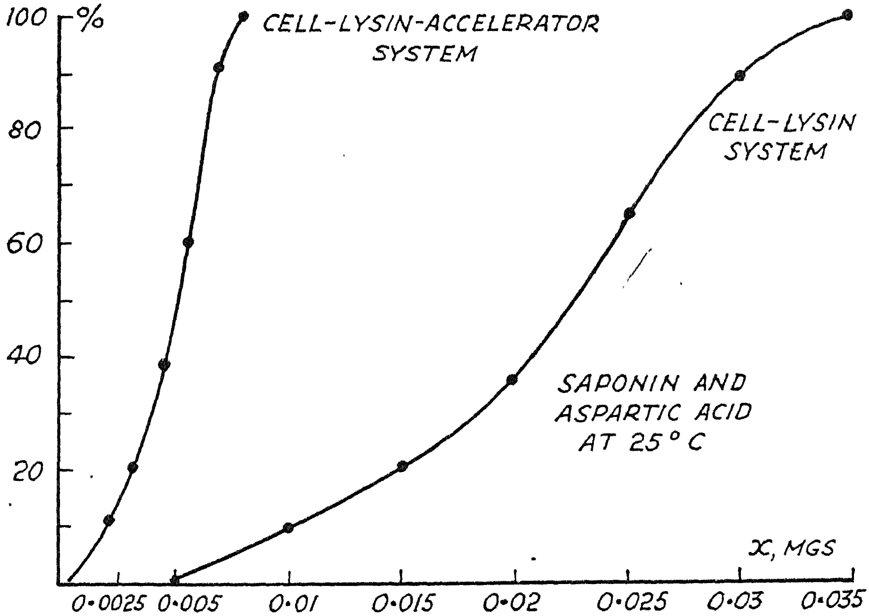

FIG. 9.

the same quantity of cells suspension as above (0·8 c.c.) and 1 in 6,250 aspartic acid, was next determined, and was found to be :—

Time.	Percentage lysis.	Time.	Percentage lysis.
0·2	11	0·5	60
0·3	20	0·6	91
0·4	38	0·75	100

Analysed in the same way as the curve for the saponin-cell system, this curve gives the integral of the frequency distribution :—

Lysin used up, x in mgs.	Percentage lysis.	Lysin used up, x in mgs.	Percentage lysis.
0·0023	11	0·0057	60
0·0032	20	0·0070	91
0·0046	38	0·0080	100

This curve is also shown in fig. 9. To analyse the acceleration, let x_2 be the amount of lysin used up in producing lysis of any percentage of the total population of cells in the saponin-cell system, and x_1 the amount used up in bringing about the same percentage lysis in the saponin-cell-accelerator system. We have then, by comparison of the curves in fig. 9, the following corresponding values of x_1 and x_2 :—

x_1 ..	0·0080	0·0070	0·0057	0·0046	0·0032	0·0023
x_2 ..	0·0350	0·0310	0·0240	0·0205	0·0150	0·0105

Plotting x_1 against x_2, we get an excellent straight line, with a slope of 0·23.

Referring to a paper previously published (4), it is to be observed that we can find the resistance constant, R, for the saponin-cell-accelerator system as compared with the saponin-cell system by taking the times for complete hæmolysis of the population of the two systems respectively. In this case we have c_1, the concentration of lysin initially added, as 0·05, and the time for complete lysis of the saponin-cell system 5·0 minutes; we also have c_2, the concentration of lysis which appears to be acting in the saponin-cell-accelerator system, as 0·218, with a time for complete lysis of 0·75 minutes. The ratio c_1/c_2, or 0·23, is equal to R, the resistance constant.

But when the resistance constant is measured in this way, it refers not to the entire population of cells, but to the most resistant classes only, for complete lysis takes place only when the most resistant cells have undergone lysis. We see, however, from the linear relation between x_1 and x_2, as obtained above, that the resistant constant which applies to the most resistant groups applies also to all other groups of the population; the effect of the accelerator is to reduce the resistance of every cell to a constant fraction of its initial resistance, so that $x_1 = R \,.\, x_2$. The way in which this reduction in resistance is effected is as yet unknown, but such evidence as there is points to it being the effect of some combination between accelerator and the cell component affected by the lysin.

Proceeding in a similar way with a concentration of aspartic acid of 1 in 12,500, and the same dilution of lysin as above, we find the integral of the frequency distribution:—

Lysin used up, x in mgs.	Percentage lysis.	Lysin used up, x in mgs.	Percentage lysis.
0·005	5	0·020	87
0·010	17	0·025	97
0·015	35	0·026	100

The ratio 0·035/0·026 gives a value of R of 0·74, which value applies to every class of the population in a very satisfactory manner.

2. *Saponin and Glutaminic Acid.*

Using a dilution of saponin of 1 in 50,000, the following integral of the distribution was obtained in the same way as above:—

Lysin used up, x in mgs.	Percentage lysis.	Lysin used up, x in mgs.	Percentage lysis.
0·005	0	0·025	75
0·010	2	0·030	94
0·015	13	0·0325	100
0·020	42		

Adding to the system a concentration of glutaminic acid of 1 in 6,250, the following integral of the distribution was obtained:—

Lysin usd up, x in mgs.	Percentage lysis.	Lysin used up, x in mgs.	Percentage lysis.
0·005	3	0·020	74
0·010	9	0·025	97
0·015	30	0·026	100

From the two curves we have the following corresponding values of x_1 and x_2:—

x_1 ..	0·026	0·0234	0·020	0·0164	0·0114	0·008
x_2 ..	0·0325	0·030	0·025	0·020	0·015	0·010

From these relations it will be seen that the value R = 0·8 applies not only to the most resistant group, but also to all other groups of the population.

The effect of other accelerators of the same type as aspartic and glutaminic acids may be explained in a similar way; in every case which we have examined we have found that the accelerator reduces the initial resistance of each class of cell by a constant fraction, this fraction being measured by the value of R.

(*b*) The interest attached to the effect of adding an accelerator to a hæmolytic system after the commencement of the reaction is an indirect one, and this case is studied only for the following reason:—We have already shown (2) that serum, if added to a hæmolytic system in which the lysin is one of the bile salts and in which the reaction has commenced, acts as an accelerator. It has also been shown that the same phenomenon occurs, to a modified extent, in a system in which the lysin is sodium oleate, and in a later paper it will be demonstrated that the acceleration observed in such systems is very similar to that observed in systems containing brilliant green and other hæmolytic dyes, when serum is added after combination between the cell component and the dye has occurred. The occurrence of this acceleration has been dealt with by Mackie (5); the similarity between the phenomena so accurately described by him and the acceleration produced by serum in taurocholate-cell systems will be seen at once by the reader. As the acceleration brought about by serum in such systems is both very important and very difficult of analysis, we think it best to consider the effect of adding an accelerator, such as glutaminic acid, to a saponin system, in which the reaction between lysin and cell component has proceeded to some extent, before attempting the more complex case of serum acceleration in a taurocholate or brilliant-green system.

In the experiment to be recorded below, we shall allow lysis to proceed in a saponin-cell system at 25° C. until 50 per cent. lysis is completed; a small quantity of accelerator will then be added, and the remainder of the reaction followed. The volume of fluid in which the accelerator is contained must be small, in order that the lysin in the system shall not be appreciably diluted by the addition; at the same time the amount of accelerator added must be great enough to markedly affect the velocity of the reaction.

Selecting as lysin a dilution of saponin of 1 in 50,000 the integral of the frequency distribution was found to be that given in experiment 2 under (*a*) of this section. When $x = 0 \cdot 021$, which value corresponds to $t = 4$, 50 per cent. of the cells in the system are hæmolysed.

Sufficient glutaminic acid, contained in a volume of 0·16 c.c. of saline, to give a dilution of 1 in 2,500 of accelerator in the system, was then added at $t = 4$, by which time 50 per cent. of the cells in the system were lysed by the saponin. The integral curve was now found to be :—

Lysin used up, x in mgs.	Percentage lysis.	Lysin used up, x in mgs.	Percentage lysis.
0·005	0	0·022	64
0·010	2	0·023	76
0·015	13	0·024	86
0·020	42	0·025	94
0·021	50	0·026	100

The two curves, that for the saponin-cell system and that for the saponin-cell-accelerator system, are shown in fig. 10. The curve for the saponin-cell-accelerator system commences at $x = 0 \cdot 021$, for at this point the glutaminic acid was added. Proceeding as in examples under (*a*) of this section, we plot

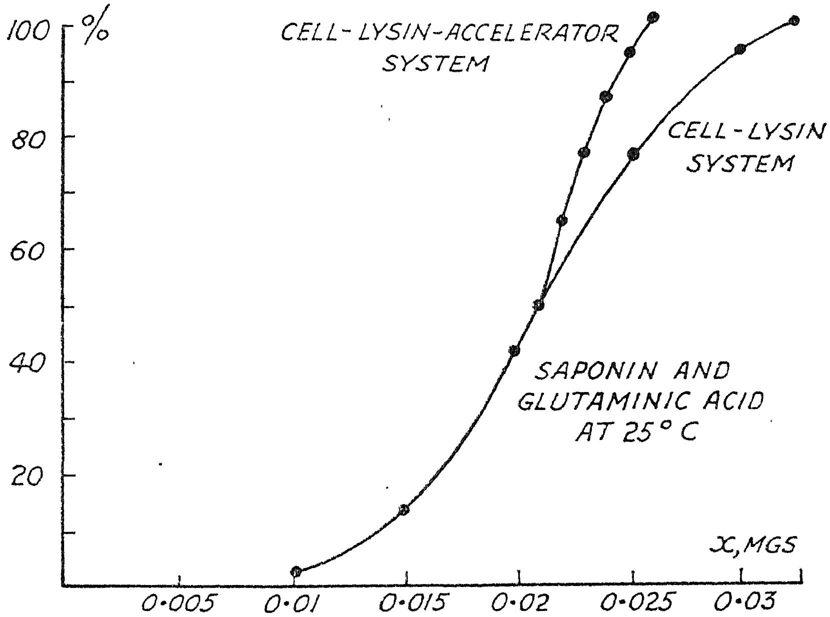

FIG. 10.

x_1 against x_2, the result of which is shown in fig. 11. Up to $x = 0 \cdot 021$ the slope of the line is unity, for the accelerator is not present; after this point, however, the relation between x_1 and x_2 is represented by a straight line with a slope of 0·5. If, then, we take the point $x = 0 \cdot 021$ as origin, we can say that the action of the accelerator is described by the expression $x_1 = \text{R} \, . \, x_2$, or, what is the same thing, that the accelerator reduces by a constant fraction the resistance of that portion of the cell component which is uncombined with the lysin at the moment of the addition of the accelerator. The case in which the accelerator is added during the reaction is thus very like that in which it is present from the beginning; the essential point is that only that portion of the cell component which is uncombined with the lysin appears to be affected by the presence of the accelerator.

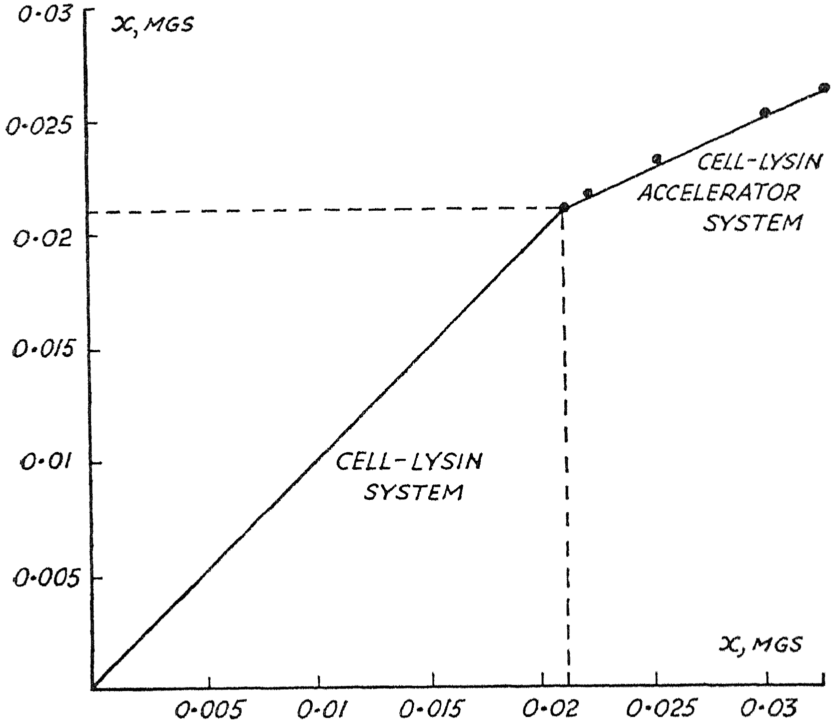

Fig. 11.

Similar results can be obtained with any accelerator of the same type as aspartic and glutaminic acids; for example, with acetic acid, with histidine, or with histamine. The only difficulty lies in the following of the very rapid reactions; apart from this, satisfactory curves are readily enough obtained.

Sometimes, with rapid reactions, only three or four points on the integral of the frequency distribution are to be found from one experiment; another experiment, however, with the observations made at different intervals, provides another three or four points, and so the whole curve may be built up.

IV.—CELL-LYSIN-INHIBITOR SYSTEMS.

Compared to the cell-lysin-accelerator systems, these are of little importance; they may, however, be considered briefly. As in the case of the systems dealt with above, the best results are obtained by allowing the cells to remain in contact with the inhibitor for some time before the addition of the lysin.

Selecting as lysin a dilution of saponin of 1 in 40,000, the integrals of the frequency distributions to the saponin alone and to the saponin together with 1 in 1,250 arginine were found as follows :—

Lysin used up.	Percentage lysis standard system.	Percentage lysis arginine system.
0·005	0	0
0·010	2	0
0·015	18	6
0·020	48	18
0·025	85	36
0·030	94	70
0·035	100	88
0·040	—	95
0·046	—	100

These two curves are shown in fig. 12. The ratio 46/35 gives R = 1·3.

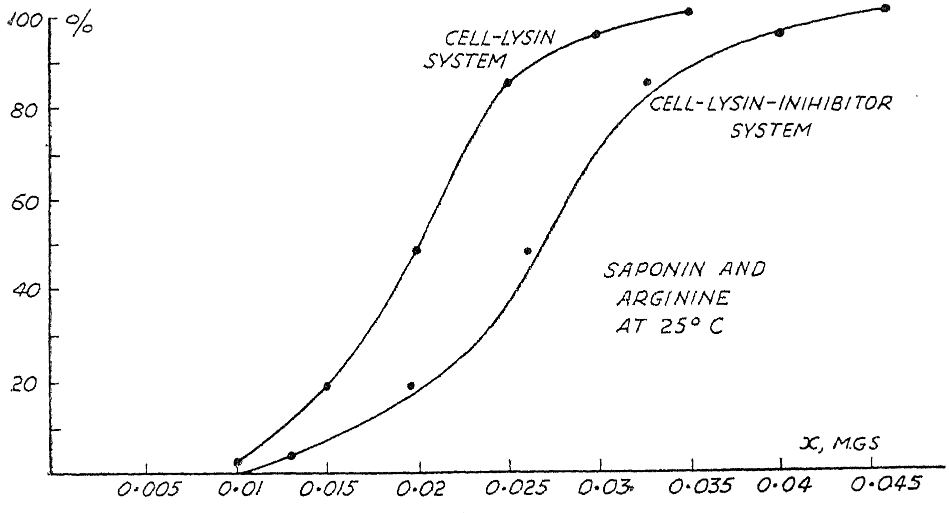

FIG. 12.

The points on the curve for the cell-lysin-inhibitor system, calculated from this value of R, are shown in the figure; they lie very closely on the experimental curve. We have, accordingly, as in the case of the accelerators, the expression $x_1 = R \cdot x_2$ to describe the relation of the two percentage hæmolysis curves.

This expression is applicable to all cell-lysin-inhibitor systems which we have examined, provided that the inhibitor is of the same type as arginine (*e.g.*, sucrose, glucose, sodium hydrate). Although, owing to the relative slowness of the reaction, experiments with such systems are more easily carried out than with cell-lysin-accelerator systems, the observations must be very carefully made, for it appears almost impossible to obtain values of R greater than about 1·4; a relatively small error in the time for complete lysis in the two systems to be compared accordingly results in an apparently very bad fit of the calculated and the observed results, and makes the experimental curve for the cell-lysin-inhibitor system appear to run parallel to that for the standard system. We mention this fact as a common source of difficulty in the analysis.

The case in which an inhibitor is added after the commencement of the reaction is similar to that in which an accelerator is added after the commencement of the reaction. The expression $x_1 = R \cdot x_2$ applies, but x_1 and x_2 are measured from the point of addition of the inhibitor as origin. We do not give curves for such cases as they are of relatively little interest, and as the effect of the inhibitor on the reaction does not differ in any essential from its effect if added at the commencement of the reaction; in each case, the cell component which is uncombined with the lysin appears alone to be affected by the inhibitor.

The quantitative results obtained by a consideration of these percentage hæmolysis curves for systems containing inhibitors and accelerators are quite in keeping with the findings recorded in (4). There it was shown that there is strong evidence for believing that the accelerators and inhibitors of the type now under consideration produce their effect by interacting with that component of the cell which unites with the lysin. The present results completely bear out this idea, and it would be very difficult to explain them on any other hypothesis. The exact mechanism by which the result is brought about is, of course, as yet unknown.

SUMMARY.

This paper is devoted to a detailed analysis of the form of the percentage hæmolysis curves for the simple lysins, acting alone, with accelerators, and with inhibitors.

Two methods for investigating the curves are described; the first is a modi-

fication of the radiometer method published in 1924, and the second one using the resistance of selenium as a measure of the light passing through the cell suspension.

It is shown that the form of the percentage hæmolysis curves for systems in which the lysin is acting alone can be exactly explained by the assumption that a monomolecular reaction proceeds among a population whose resistances are distributed according to a frequency curve, usually of Pearson's Type II. The lysins dealt with are saponin and sodium taurocholate; the method of analysing the curves is given in full, with examples from experiment.

The cases where (*a*) an accelerator is added to the system before the reaction begins, (*b*) an accelerator is added after the reaction has commenced, (*c*) an inhibitor is added at the commencement of the reaction, and (*d*) an inhibitor is added after the reaction had begun, are considered. It is shown that the effect of the accelerators and inhibitors is to alter the resistance of the population to the lysin, the alteration of resistance being described by an expression containing the single resistance constant, R.

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The Viscous Elastic Properties of Muscle.

A. Levin* and J. Wyman.†

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(From the Department of Physiology and Biochemistry, University College, London.)

I. *Introduction.*

Towards the middle of the last century Weber (1) investigated the problem of muscular contraction from the physical and mechanical points of view. He thought it possible to express the activity of the muscle solely in terms of a change in its elastic condition in response to stimulation. In other words, he assumed that the active muscle behaved as a stretched spring and that the tension-length curve of a given muscle, under a definite set of external conditions, was invariable and independent of the particular way in which it was obtained. This assumption was shown to be erroneous by Blix (2), with the help of his myographion, by means of which tension-length diagrams both of shortening, and of stretched, muscle could be automatically recorded. Blix showed that, at any length, tension developed during stretch was greater than corresponding tension developed during release. This observation was confirmed and extended by Fick (3, 4), who at first assumed an increased " physiological " response in stretch as the cause of this effect. In order to measure this response he determined the *total* energy of a contraction, this term denoting the algebraic sum of mechanical work and heat produced by the muscle. This quantity was found to be *smaller* in the case of a stretched muscle, and no satisfactory explanation was available capable of fitting the more elaborate picture which had emerged, largely from Fick's hands.‡

A new aspect of the problem was created by the work of A. V. Hill (5) and his associates (6, 7) from twenty to thirty years later. These investigators introduced the concept of friction and viscosity, irreversibility in the thermodynamic sense, into the study of muscle. The effect of this point of view was immediate. In Hill's paper of 1922 experiments were described which promised to clear away a substantial part of the difficulties presented by the work of Fick (and others). Hartree and Hill (8), in a paper on thermoelastic phenomena in muscle, had previously shown that " when the length of a muscle is altered passively, a considerable quantity of heat is evolved, which is greater the more sudden the change of length." They had attributed this phenomenon to the degradation of a portion of the mechanical energy of the system into heat as a result of frictional and viscous processes accompanying deformation; and Hill expressed the view that deviations from the theoretical maximum work (as calculated from isometric tensions developed at various lengths) and the actual work obtained when a frog's muscle was allowed to shorten by means of a maximum work device (see Doi (9)) were to be ascribed to the same cause, *i.e.*, wastage of work in the viscous material of the muscle itself. In 1922 Hill (5) investigated the matter in the case of human arm muscles by having the arm to work against the inertial reaction of a heavy fly-wheel, the mechanical advantage and, consequently, the speed at contraction being variable. It was found that the work decreased with increase in the speed of shortening, as expressed by the formula

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* Working for the Medical Research Council.

† Fellow of the National Research Council, U.S.A.

‡ We have omitted any account of the work of various other investigators in order to emphasise the main trend of the problem with which we are concerned in this paper.

$$W = W_0\left(1 - \frac{k}{t}\right),$$

where W is the work, t is the time occupied by the shortening, and W_0 and k are constants. Now this is precisely the expression for the work done by a simple viscous-elastic system shortening at any constant speed $1/t$ (see below). In this case W_0 gives the ideal work which the system is capable of doing as the speed of shortening approaches zero, and k is a constant depending on the viscosity.

A wider development of this idea was contained in the paper by Gasser and Hill (10). In this paper the results obtained with human arm muscles were confirmed in general by experiments on the directly stimulated sartorius of the frog. Further experiments also were carried out on the effects of sudden stretches and releases on the tension registered by frog's muscle. It was found that after an " instantaneous " release of an isometrically stimulated muscle, there was a drop of tension below the normal isometric value for the new length, proportional to the percentage shortening. A 15 per cent. shortening caused complete disappearance of tension. Such a drop of tension was followed by a redevelopment along the same curve (when tension was plotted against time) as that for the development of an isometric contraction. When the shortening was carried out more slowly, the drop of tension was less marked, until, for very low speeds, the tension fell gradually from the isometric value for the initial to that for the final length. If, on the other hand, the muscle was stretched, there was a momentary rise of tension depending on the extent and suddenness of the stretch. If the stretch was very sudden, the rise was followed by a fall, and that in turn by a redevelopment to the tension characteristic of the new length along the usual curve for an isometric contraction. If the stretch was less sudden, the tension rose above the value for the new length and approached equilibrium from above. When a quick release was followed by a quick stretch, the initial tension was at once restored, and similarly for a quick stretch followed by a release. All these rather complicated results Gasser and Hill attributed to the viscous properties of the muscle system, which prevented the immediate attainment of equilibrium when its length was changed. They also made direct determinations of changes in viscosity consequent upon stimulation by means of recording the damping effects of muscles on the oscillations of a flat steel spring. Finally they concluded their experiments by showing that, qualitatively at least, their results could be reproduced with a viscous-elastic model described in their paper.*

If, thus, the introduction of the concept of viscosity removed many of the objections arising from the work of Fick and others, there still remained the question of the total energy liberated by the muscle. Fick's experiments dealing with this matter did not admit any such simple interpretation. His heat measurements have been recently substantiated by the researches of Fenn (11, 12) and Wyman (13). Fenn had, it is true, confined his attention in the main to cases of shortening muscles ; but Wyman had made a careful study of stretches as well, using the improved thermal methods of Hill for measuring heat and a new and very accurate form of myograph for recording work. He found, just as Fick had done, that the muscle liberated more total energy when it shortened and did external work than when it was stretched and work was done on it. Wyman, however, pointed out that the values of the total energy calculated from the protocols of all these experiments were based on a certain assumption, which, though plausible, was by no means assured. It was taken for granted in the calculations that the chemical energy liberated in contraction (the measure of the physiological response) all appeared either as work or heat ; in particular it was assumed that any mechanical potential energy present at the moment of relaxation would be degraded quantitatively into heat. Unless this assumption were granted (if, for example, it were supposed that part of the mechanical energy disappeared directly as chemical energy in relaxation), it was wholly impossible to say, from the results of these experiments, whether more energy was liberated in a stretch or in a shortening, and it was quite reasonable to assume that it was the same for both. It appeared possible, therefore, that the total energy liberated by the muscle was independent of the conditions of work under which contraction took place, and that the simple *viscous-elastic* view of muscle was, after all, correct.

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* Hill himself has recently repeated and confirmed in the case of *Holothuria nigra* and tortoise the part of these experiments dealing with stretches and releases. See Hill (10).

What was needed, in the opinion of the writers, in view of the complexity of the situation, was further work along two lines. In the first place it was important to carry out fresh and more searching experiments on the viscous-elastic properties of muscle, following the lead taken by Hill. In the second place it was necessary to find independent ways of studying the energy liberation of muscle under different conditions of shortening and lengthening in order to determine whether or not there really are corresponding differences in "physiological" response. Experiments of the first type form the subject of the remainder of this paper.

II. *Method.*

The general plan of the experiments on the mechanical properties of muscle has been to obtain really accurate tension-length curves for stretches and releases at different constant speeds, and to study these curves quantitatively as far as possible, subjecting them to careful analysis with the aid of various types of viscous-elastic models. In this way it was hoped to throw further light on the real nature of muscle regarded as a viscous-elastic system.

In order to attain this end we have made use of a new type of myograph, previously described in another connection (13), but for this purpose somewhat modified and entirely overhauled. This instrument differs substantially in design from the Blix's myographion. Whereas in the latter it is the rate of change of *tension* which is within the control of the experimenter and is kept approximately constant (so far as this can be done by merely manual means), the corresponding change of length being recorded by the muscle itself, in the instrument described below it is the *length* which is changed at a constant rate (this being achieved by mechanical means), the corresponding change of tension being recorded by the muscle. The final result for both instruments is the tension-length diagram. In such a diagram the work done by, or on, the muscle is given by the area enclosed by the curve. Curves obtained by our instrument are given below in fig. 2 and others.

The construction of the machine, with the latest improvements, is represented in fig. 1. The muscle M is supported in a vertical position, its lower end held immovable by the clamp C and its upper end connected by an inextensible wire with an isometric lever L. This lever is mounted on a bar B, which may be rotated in the vertical plane about the axis A. In this way, by suitably suspended weights W, either a stretch or a shortening may be carried out. The extent of movement is controlled by adjustable stops S. The speed is regulated by the needle valve V in the piston of the dashpot P, and may be varied from zero to about 50 cm. per second; it is measured by recording on the indicator diagrams the vibrations of a pneumatic tambour (not shown on the diagram) mounted on the bar and reproducing the oscillations of a vertical pendulum of period 1/2 to 1/50 sec. The arrangement of the dashpot ensures that constant speed is reached almost instantaneously and maintained throughout the movement. Release of the bar at any desired moment is effected by closing the circuit of the electromagnet E, which controls a catch mechanism consisting of a tooth T gripping a notched plate N mounted in the end of the bar B. It is essential that both shortening and lengthening take place between the same limits, and this is ensured by having the bar move always the full distance between the stops.

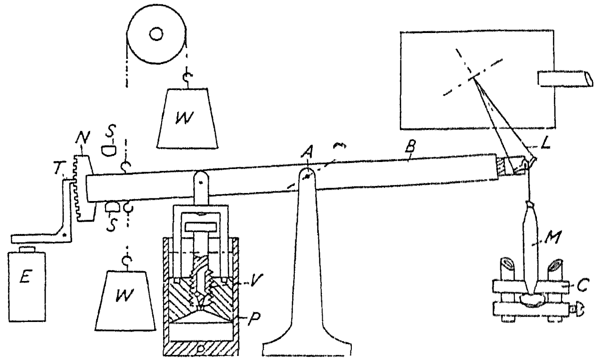

Fig. 1.—*Diagram of instrument for recording tension-length curves of muscle.* (See text.)

In the course of the experiments it soon became evident that special precautions were required in order to obtain records free from mechanical distortion. Even a very small amount of "give" in the arrangement, for example, in the lever L, due to its not being perfectly isometric, or in the clamp C, was found to influence the shape of the curves and make analysis uncertain, on account of the difficulty of allowing for effects extraneous to the muscle. Accordingly special attention was given to making the lever as isometric as possible. Moreover, to obtain the greatest possible rigidity combined with the least moment of inertia, the rocking bar B was made of a straight piece of hollow steel tubing. In order that the tension-length axes of the diagrams should be perpendicular, the arm of the lever L was set obliquely to the bar. The arm itself was designed according to the principle of girder construction and given the shape of a triangle, of which the two long sides were formed of thin bamboo fibre and the short base was a light brass rider mounted directly on the spring of the lever. This arrangement possesses the advantage of having the smallest possible moment of inertia combined with the greatest rigidity in the plane of recording, at the same time being very flexible at right angles to that plane so that the friction of the tracing point is small. In order further to reduce the period of oscillation of the lever, the arm was made comparatively short (5 cm.).* The recording itself was done on an immobile smoked glass surface with a fine glass point. The curves obtained in this manner could be magnified and copied with great accuracy by means of a projection lantern, so that no inconvenience was experienced due to the small size of the records, whilst advantage was derived from almost complete absence of oscillations of the lever.

With this arrangement it has been possible to obtain very accurate tension-length curves for stretches and releases of various muscles under definitely reproducible conditions. The muscle to be studied is carefully dissected out of the animal and fixed in the clamp C. It is tied directly at its upper end by a stout thread to the wire rod, which connects it with the isometric lever of the instrument. Great care is taken to ensure that there is no length of tendon or thread intervening between the muscle and the wire, which might confuse the results by the introduction of extraneous elastic effects. The stimulus used consists of a moderately strong tetanising current passed through the entire length of the muscle. A "Harvard" induction coil was used with a 2-volt battery and the secondary coil slightly overlapping the primary. When, and not until, the muscle has developed maximum tension in response to the stimulus, the bar B is released by the tooth device T and the stretch or shortening is carried out. If the experiment extends over any considerable time, care is taken to provide that the temperature is maintained constant: in the case of the experiments on tortoise, the muscle was kept in a Dewar flask brought up from below and supported by the clamp bearing the muscle. In most cases an experiment consists of a series of operations at different speeds, starting with quick releases, proceeding to quick stretches, and then returning to quick releases. In this way the effects of fatigue, if present, can be observed and allowed for.

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* This type of lever arm proved useful to one of us (A. L.) in connection with work on recording the form of the pulse-wave (unpublished). It appears to us that it might be found applicable in all cases where good mechanical properties of the lever arm are of particular importance.

III. *Results on Dog-Fish.*

Studies have been carried out on various types of muscle with results which, in the light of theory, appear very consistent. We begin our discussion with what has proved to be the simplest case, the jaw muscle of the dog-fish. The results obtained with this muscle are best described in connection with the actual curves taken from the protocol of an experiment. Fig. 2 gives a set of tension-length curves, magnified and copied in the way we have described above, obtained from a single muscle. The individual curves were superimposed so as to bring the axis of ordinates and abscissæ into coincidence. Each curve represents a stretch or release (right- and left-hand groups respectively) at a definite constant speed. The quickest release is the curve farthest to the left; the quickest stretch that farthest to the right; the other curves lie between in the order of speed. The broken line represents the ideal curve for zero speed; the curve for the slowest release has been corrected for fatigue, as shown by the shorter broken line. The line AB, corresponding to a stretch or release at zero tension, is obtained by joining the points of zero tension at the two lengths between which movement takes place. It is evident at once that these curves afford a striking confirmation of the observations of Blix and Fick that at any given length a muscle exerts greater forces when it is being stretched than when it is shortening. Moreover, it appears that this effect is the greater the greater the speed of movement: the curves for the stretches migrate always

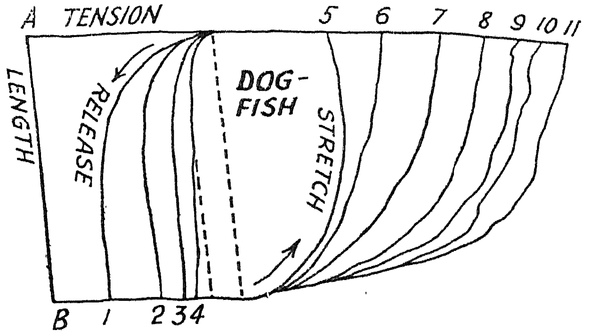

Fig. 2.—*Tension-length curves from the jaw muscle of the Dog-Fish.* Numbers indicate the order in which the observations were made. The corresponding speeds (arbitrary units) are as follows :—

(1)	...	6·15	(5)	...	0·17	(9)	...	3·47
(2)	...	0·70	(6)	...	0·25	(10)	...	3·69
(3)	...	0·27	(7)	...	0·49	(11)	...	6·71
(4)	...	0·13	(8)	...	1·42			

to the right as the speed of movement increases ; those for the releases to the left. But beyond this the *form* of the curves is very definite and, as we shall see, significant.* In the first place they are not straight lines, but are exponential in character ; there is a large initial rise or drop of tension at the beginning of movement, which falls off as the movement goes on ; and in their subsequent course curves for different speeds of stretch or release tend to become parallel. In the second place the curves for the stretches or releases all start out in coincidence, cutting the line of isometric development of tension at a definite finite angle. (See also fig. 10*b*.)

Fig. 3 contains results of a more indirect kind, but no less important. In this curve work done by the muscle, or in practice values proportional to this work, obtained by measuring the areas of the tension-length curves of fig. 2, are plotted against speed of movement. Since we are interested only in absolute values, we neglect the difference in sign for work done in stretches and releases. On the other hand, in plotting speed we preserve differences in sign and take stretches as positive (displacement increasing with time) and releases as negative. The resulting curve is S-shaped : it is continuous through the axis of ordinates and it appears to have an upper and a lower asymptote, the

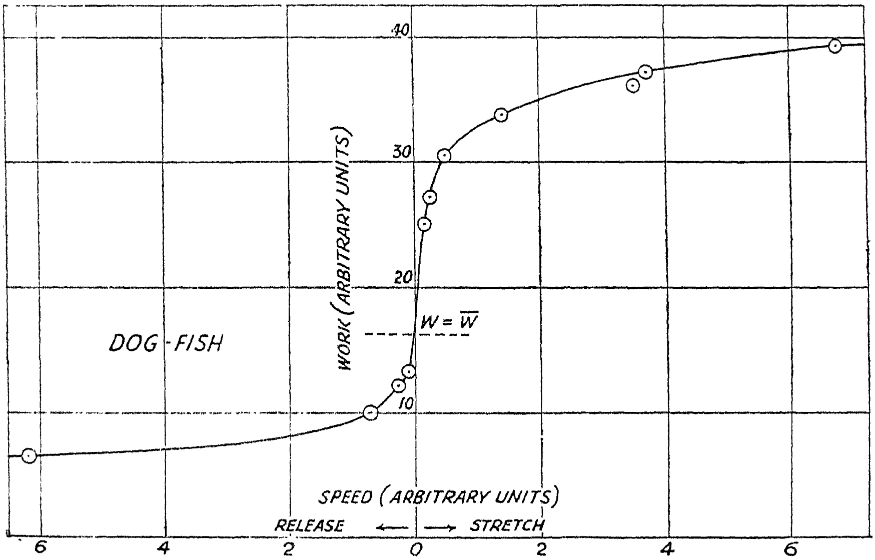

FIG. 3.—*Work-speed curve* plotted from the results shown in fig. 2.

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* It must be pointed out that in these experiments the recording arm of the lever was not always adjusted so as to give rectangular co-ordinates, but since we are only interested in relative values of force and work, we may neglect this.

former for the right and the latter for the left-hand branch, both parallel to the axis of abscissæ. This indicates the presence of an upper and a lower value for the work done by or on the system, approached as the speed of movement becomes very great.

IV. *Models.*

These results are of considerable interest as they suggest the presence in muscle of an element of free or undamped elasticity, a point which will be discussed at length below. At the same time they render untenable Hill's view in its original form. For the equation given above (p. 219) used by Hill to describe the behaviour of human arm muscles, and on which the original viscous-elastic theory was based, implies that muscle is, mechanically, a system of the type illustrated by Model I, *i.e.*, a single spring damped at its free end by a vane surrounded by a viscous fluid (fig. 4); whereas, in fact, this system may be shown to give results quite different from those we have just described for muscle. For example, it is obvious that Model I, when being stretched or allowed to shorten, will exert forces greater than or less than those due to the spring alone by an amount proportional to the speed of movement. From this it follows that at the beginning of a release at any finite speed the tension should drop (or rise in the case of a stretch) *instantaneously*, an effect which is not found in the case of muscle. Furthermore, the tension-length curves for stretches

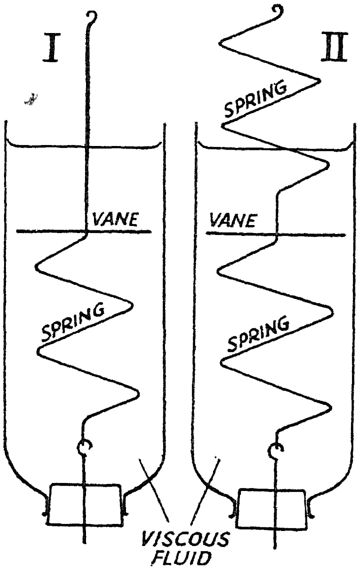

Fig. 4.—*Viscous-elastic models* to illustrate the behaviour of muscle. (See text.)

and releases should be throughout strictly parallel to one another and to the ideal curve corresponding to zero speed of movement; but this latter curve is for muscle as well as for Model I approximately a straight line, as may be inferred from tensions developed isometrically at intermediate lengths and as has indeed been shown directly for tortoise muscle by actually carrying out very slow releases or stretches (see fig. 10*b*). The curves of our tension-length diagrams, on the other hand, are far from being straight lines. Moreover, since work is simply the integral of force with respect to the distance passed through, the work done by Model I or any system of the same type will be a linear function of the speed. We have seen that this is not the case for muscle.

It might be supposed that this discrepancy between the results actually recorded for muscle and those predicted for a system similar to Model I arises from methodical errors on our part rather than any fundamental differences of structure. It is to be expected, for instance, that any element of free elasticity, even very small, introduced into the arrangement will of itself give rise to effects of the kind observed (see below). Great care in the preparation of the muscle makes it possible to eliminate at once the question of elasticity of the tendons or of the thread used in fastening the muscle to the wire. It would, however, be difficult to rule out the possibility of effects due to "give" in the recording instrument itself or in the clamp without an experimental test of the matter. For this purpose an actual model of the type we have just been discussing (Model I) was constructed and substituted for the muscle. The results obtained in this way are shown in fig. 5. The tension-length curves for greater speeds

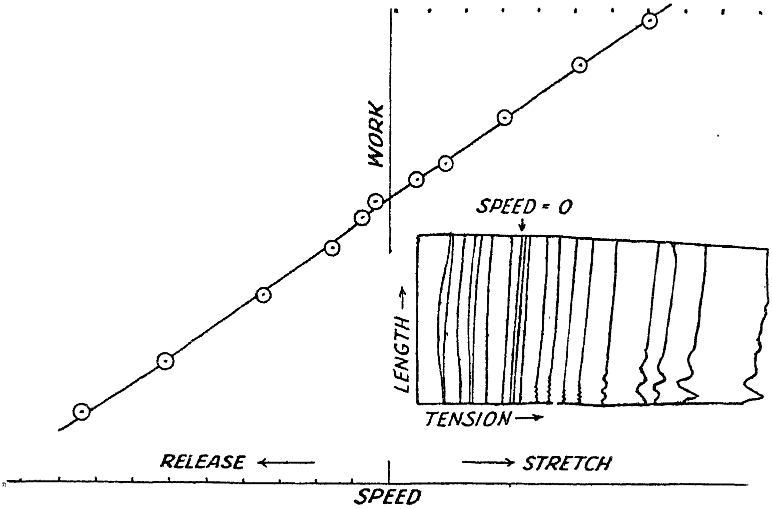

FIG. 5.—*Tension-length and work-speed curves from Model I.*

are somewhat marred by oscillations which were very difficult to avoid with this arrangement,* but both they and the work-speed curves obey exactly the behaviour we have predicted above. This makes it clear that the discrepancy we have been discussing is genuine, and that the effects observed in the case of muscle are inherent in its structure.

We have already remarked that the tension-length and work-speed curves suggest the presence in muscle of an undamped elastic element in addition to a viscous-elastic structure of the type of Model I. We now propose to examine and justify this hypothesis with the aid of a second mechanical model (II) represented in fig. 4.† This model differs from Model I in having another spring attached above the damping vane. We thus have a *two-part viscous-elastic system of which one elastic element is free and the other damped.* We shall show the essential identity of behaviour of this model and muscle.

It is not difficult to deduce from mechanical considerations the behaviour of Model II when it is subjected to stretches and releases of the type carried out with muscle. What we desire is an expression for F, the force exerted by the free end of the top spring, as a function of its displacement, up or down, from the initial position, for any given velocity of movement. From this we may determine the character of the tension-length curves for the system. In the treatment which follows it is to be noted that since we are dealing with a system which is capable of compression as well as extension, unlike many analogous systems which buckle (such as muscle), the results hold for positive and negative values of all the variables.

Let α_1 and α_2 refer to the elastic coefficients of the lower and upper springs (lengths l_1 and l_2) respectively, similarly x_1 and x_2 to the extensions of the same when the system is stretched; let η be the viscosity, or, strictly speaking, the total damping effect of the vane. We assume that the inertia of the arrangement is negligible; consequently we may equate the opposing forces acting at the point of attachment of the vane and write as the fundamental equation describing the motion of the system

$$(1) \qquad \frac{\alpha_1}{l_1} x_1 + \eta \frac{dx_1}{dt} - \frac{\alpha_2}{l_2} x_2 = 0,$$

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* These oscillations are not due to the recording lever, as might be supposed. The connection between the lever and the damping vane being absolutely inextensible and the lever itself very isometric, any minute vibrations of the steel bar supporting the lever, set up in the moment of stretch, were reproduced on a magnified scale.

† This model has been discussed by Poole in connection with natural fibres (14). The paper of Shorter (15) is also of interest in this connection.

which gives, when we differentiate with respect to the time,

$$\text{(2)} \qquad \frac{\alpha_1}{l_1}\frac{dx_1}{dt} + \eta\frac{d^2x_1}{dt^2} - \frac{\alpha_2}{l_2}\frac{dx_2}{dt} = 0.$$

Now the net force F exerted by the free end of the top spring, which would be recorded by our instrument if the model were substituted for the muscle, is

$$\text{(3)} \qquad \mathrm{F} = \frac{\alpha_2}{l_2}x_2.$$

Furthermore, during any stretch or shortening carried out at constant speed v, we have

$$\text{(4)} \qquad \frac{dx_1}{dt} + \frac{dx_2}{dt} = v.$$

Eliminating x_1 and x_2 between equations (2), (3) and (4) we obtain

$$\frac{d^2\mathrm{F}}{dt^2} = \frac{\alpha_1/l_1 + \alpha_2/l_2}{\eta}\cdot\frac{d\mathrm{F}}{dt} = \frac{\alpha_1\alpha_2}{l_1l_2}\cdot\frac{v}{\eta},$$

of which the complete solution is, as may be shown by substitution,

$$\mathrm{F} = \mathrm{A} + \mathrm{B}\left(1 - e^{-\frac{(\alpha_1l_1+\alpha_2/l_2)}{\eta}t}\right) + \frac{\alpha_1\,.\,\alpha_2vt}{\alpha_1l_2 + \alpha_2l_1}.$$

The value of the constants A and B may be obtained from the two relations. (i) that when $t = 0$, $\mathrm{F} = \mathrm{F}_0$, where F_0 is the tension exerted by the system in equilibrium at the initial length; and (ii) that also, when $t = 0$, the velocity of the vane is zero, *i.e.*, from equation (4),

$$\frac{dx_1}{dt} = 0 = v - \frac{dx_2}{dt},$$

whence from equation (3)

$$v = \frac{l_2}{\alpha_2}\frac{d\mathrm{F}}{dt}.$$

When A and B are worked out and we substitute for t the quantity y/v, where y is the displacement of the free end of the top spring, positive or negative according to the sign of v, from the initial position, we arrive at the result

$$\text{(5)} \qquad \mathrm{F} = \mathrm{F}_0 + \frac{(\alpha_2{}^2/l_2{}^2)\eta v}{(\alpha_1/l_1 + \alpha_2/l_2)^2}\cdot\left(1 - e^{-\frac{(\alpha_1/l_1+\alpha_2/l_2)}{\eta v}y}\right) + \frac{\alpha_1\alpha_2/l_1l_2}{\alpha_1/l_1 + \alpha_2/l_2}y,$$

or, since the sum of the first and last term on the right gives the "reversible" tension $\bar{\mathrm{F}}$ corresponding to infinitely slow movement,

$$\text{(6)} \qquad \mathrm{F} = \bar{\mathrm{F}} + \frac{(\alpha_2{}^2/l_2{}^2)\eta v}{(\alpha_1/l_1 + \alpha_2/l_2)^2}\left(1 - e^{-\frac{\alpha_1/l_1+\alpha_2/l_2}{\eta v}y}\right).$$

It is obvious that the relation between $\bar{\mathrm{F}}$ and y is linear. Consequently the tension-length curves of the model are of an exponential character, when referred to a base line corresponding to infinitely slow movement, and agree in type with those obtained from muscle. The theoretical case differs, however, from the case of muscle in so far as it includes negative tension (compression) of either or both of the springs, the analogue of which is obviously impossible in muscle. But in the actual model there is no such discrepancy, provided the connections between the springs and the recording lever, etc., are flexible and therefore liable to buckle, or provided the springs themselves are represented by rubber bands.

We may emphasise two points of very definite agreement between two sets of curves. (*a*) In the first place it is seen that the force F exerted by the model drops or rises along an exponential curve as shortening or lengthening proceeds. As y increases $\mathrm{F} - \bar{\mathrm{F}}$ approaches $\dfrac{(\alpha_2{}^2/l_2{}^2)\, v\eta}{(\alpha_1/l_1 + \alpha_2/l_2)^2}$ as a limit and the curve tends to become a straight line parallel to the line $\mathrm{F} = \bar{\mathrm{F}}$. This is just what we observed in the case of muscle. (*b*) In the second place

$$\frac{d\mathrm{F}}{dy} = \frac{d\bar{\mathrm{F}}}{dy} + \frac{\alpha_2{}^2/l_2{}^2}{\alpha_1/l_1 + \alpha_2/l_2} e^{-\frac{\alpha_1/l_1+\alpha_2/l_2}{\eta v}\cdot y}. \tag{7}$$

When $y = 0$ this reduces to

$$\frac{d\mathrm{F}}{dy} = \frac{d\bar{\mathrm{F}}}{dy} + \frac{\alpha_2{}^2/l_2{}^2}{\alpha_1/l_1 + \alpha_2/l_2}, \tag{8}$$

which, since $\bar{\mathrm{F}} = \mathrm{F}_0 + \dfrac{\alpha_1\alpha_2}{\alpha_1/l_1 + \alpha_2/l_2} y$ and therefore $\dfrac{d\bar{\mathrm{F}}}{dy} = \dfrac{\alpha_1\alpha_2/l_1 l_2}{\alpha_1/l_1 + \alpha_2/l_2}$, gives $\left(\dfrac{d\mathrm{F}}{dy}\right)_{y=0} = \dfrac{\alpha_2}{l_2}$, which is independent of the values of v and η. This shows that curves for different speeds all coincide at the start, intersecting the "isometric" tension line at an angle $\phi = \tan^{-1}\dfrac{l_2}{\alpha_2}$, just as in the case of muscle. Moreover, this angle is independent of any changes in viscosity such as those produced by changes of temperature. This, too, was found to be the case with muscle. (In three different sets of releases carried out with the same tortoise muscle at three different temperatures, the following values of ϕ were obtained :

$\mathrm{T} = 13{\cdot}6°$ C., $\phi = 12{\cdot}4°$; $\mathrm{T} = 7{\cdot}5°$ C., $\phi = 12{\cdot}4°$; $\mathrm{T} = 21{\cdot}5°$ C., $\phi = 12{\cdot}3°$.)

Similar agreement in the behaviour of the model and the muscle is observed when we turn to the relation of work to speed. The work done by, or on, the model in a stretch or release of extent Δy may be obtained by integration of the expression for F in equation (6) with respect to y and is given by

$$(9) \qquad \mathrm{W} = \overline{\mathrm{W}} \pm \frac{(\alpha_2/l_2)^2 \eta v}{(\alpha_1/l_1 + \alpha_2/l_2)^2} \cdot \left[\Delta y - \frac{\eta v}{\alpha_1/l_1 + \alpha_2/l_2} \left(1 - e^{-\frac{\alpha_1/l_1 + \alpha_2/l_2}{\eta v} \Delta y}\right) \right].$$

The graph from this equation, relating W and v, is an S-shaped curve of the type obtained from muscle, crossing the axis of ordinates (zero speed) at a value of $\mathrm{W} = \overline{\mathrm{W}}$ and having an upper and lower asymptote corresponding to the speeds of movement so great that the lower spring has no time to change its length at all. Both the upper and the lower limiting values of W are thus determined solely by the properties of the upper spring. The right and left branches of the curve (referred to the axis of ordinates) are strictly analogous, and either may be obtained by rotation of the other in the plane of the figure through 180° about the point ($\mathrm{W} = \overline{\mathrm{W}}$, $v = 0$).

In regard to this latter property alone there seems to be some divergence between the dog-fish and the model. In the work-speed curve for the dog-fish the two branches are not strictly analogous ; the right-hand branch (stretches) rises more above the line $\mathrm{W} = \overline{\mathrm{W}}$ than the left-hand branch falls below it. But this is easily explained when we recall that in the case of muscle we are dealing with a system in which the tension can never become negative, whereas equation (9) is valid without restriction only for systems capable of compression ; and it is easy to see that any discrepancy between the two branches of the work-speed curve for a system which buckles must be in the direction observed in the case of muscle. It may be objected here that the discrepancy in question prevails over a range of speeds within which the tension of the muscle does not actually at any point of the shortening fall to zero (see fig. 2), so that this argument cannot apply. But we must remember that in muscle we are dealing with a system of very complex microscopic structure, which may be expected to represent not a single model of the type under discussion, but rather a whole family of them arranged in parallel like the fibres, with constants distributed about certain mean values. It is then to be expected that as the speed of shortening increases the more viscous elements will be unable to follow and will buckle, though the tension exerted by the muscle as a whole is not zero. In this way it is possible for the discrepancy in question to arise without the tension of the muscle vanishing. With the removal of this difficulty, therefore, we are entitled to regard dog-fish muscle, from a mechanical point of view, as a system of the type represented by Model II.

For purposes of illustration, and to render the matter more convincing, we have actually constructed a working model like that shown in fig. 4 (II), and curves obtained from this model with our recording instrument are given in fig. 6. The general agreement in the behaviour of muscle and model is apparent by comparison of these with figs. 2 and 3.

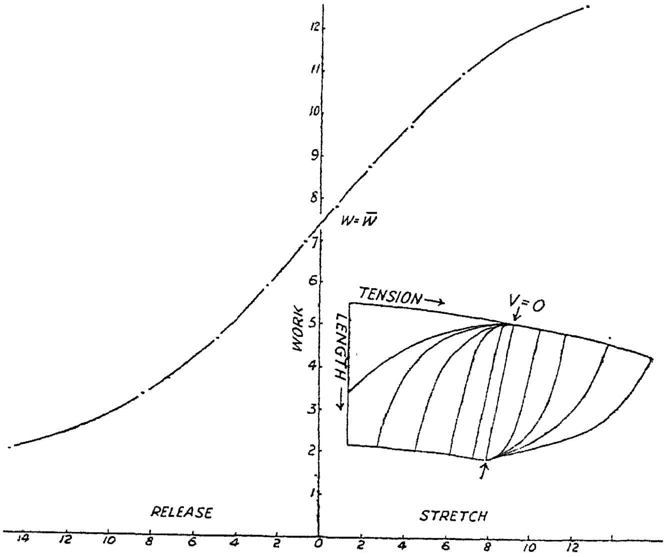

FIG. 6.—*Tension-length and work-speed curves from Model II.*

(An adjustment of the scale of speed and of the relative lengths of the two springs would turn the work-speed curve of fig. 6 into that of fig. 3.)

V. *Further Experimental Results.*

We now pass on to results obtained with other muscles. Fig. 7 refers to the longitudinal body-wall muscles of *Holothuria nigra,* and fig. 8 to the lantern muscles of *Echinus.* In both cases we are dealing with smooth muscles, which are many (perhaps 100) times slower in their response than the jaw muscle of the dog-fish; consequently the speed scale in the work-speed curves is of a different order from that of fig. 3. Both muscles are very fragile and frequently gave trouble by actually tearing during the course of an experiment. Allowance had to be made for this as for fatigue in plotting the curves, and for this reason the results are less reliable than those for the other muscles. Nevertheless, they are in very good qualitative agreement with what we have found for dog-fish.

On the other hand, the results obtained in the case of the *biceps cruris* muscle of the tortoise (fig. 10) and the claw muscle of the spider crab (fig. 9) are at first sight very perplexing. It is evident both from the work-speed and tension-length curves that speed is far less effective in increasing the tension during a

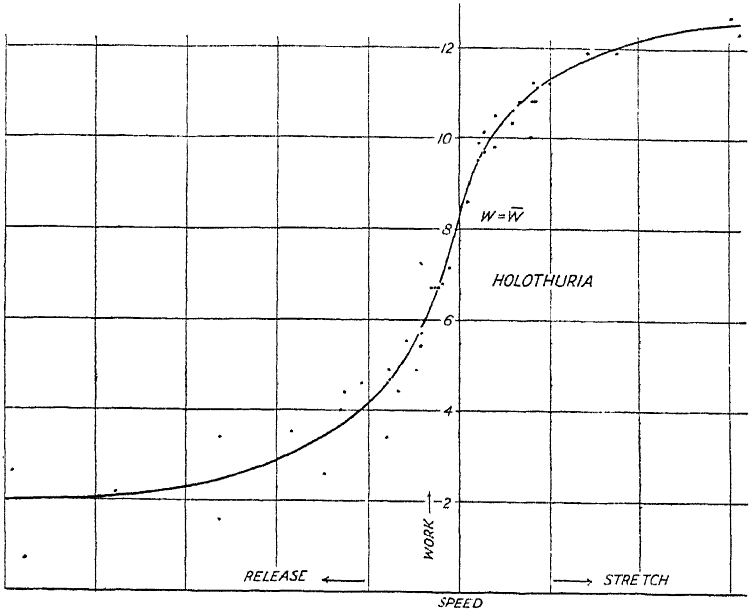

FIG. 7.—*Work-speed curve from longitudinal body muscle of Holothuria nigra*

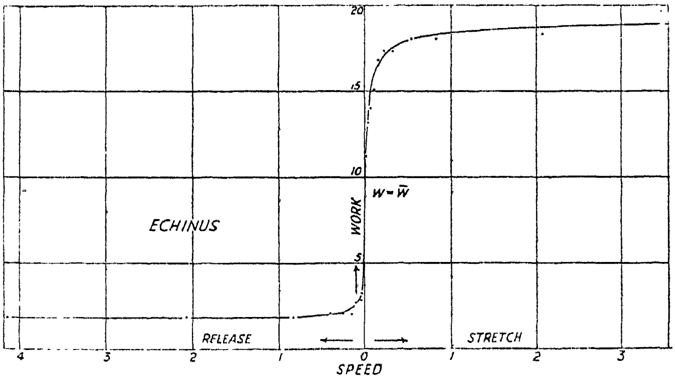

FIG. 8.—*Work-speed curve from lantern muscle of Echinus.*

stretch than in lowering it during a shortening. The discrepancy in form between the two branches of the work-speed curve is, in fact, just the opposite of what we observed for dog-fish muscle. The phenomenon cannot possibly be accounted for in terms of buckling. On the other hand, the appearance of the tension-length curves for the stretches suggests a rupture of some of the contractile elements soon after the beginning of movement and consequent rise of tension. After a rapid initial rise of tension the curves for the stretches

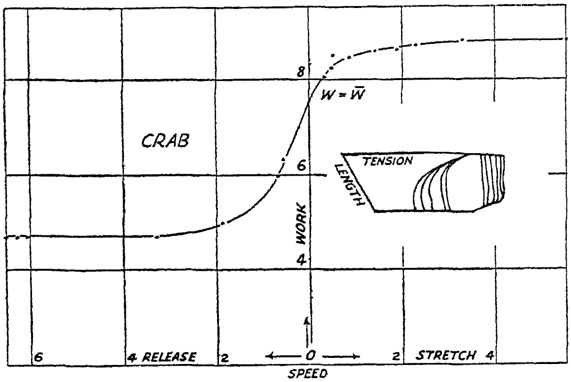

Fig. 9.—*Tension-length and work-speed curves from Spider Crab.*

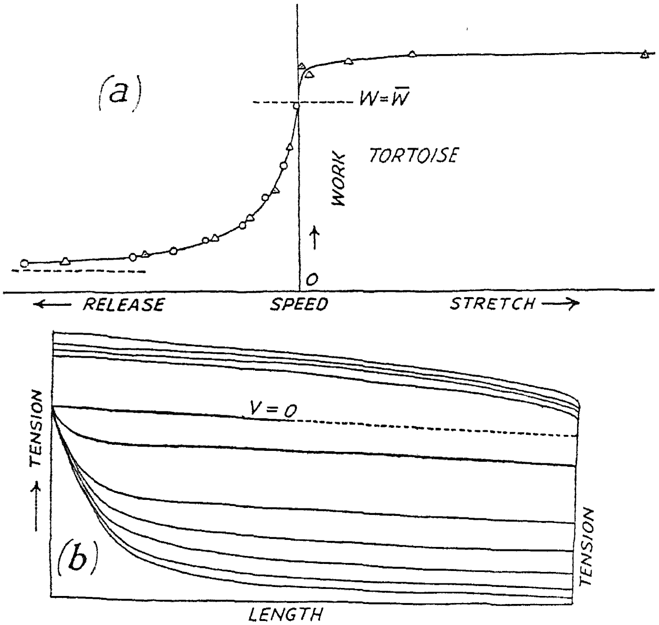

Fig. 10.—*Tension-length and work-speed curves from Tortoise.*

become somewhat irregular and frequently show oscillations. This corresponds to a point of discontinuity beyond which stretching produces comparatively small further rise of tension. The muscle behaves as if it were unable to develop more than a certain limited amount of tension, and as if slipping or breaking of some elements occurred each time it was stretched beyond this limit. The breaking or slipping may be supposed to go on as stretching proceeds ; it cannot, however, result in any permanent, irreversible damage to the muscle, for tensions developed in subsequent contractions are as high as ever. Such breaking possibly involves certain molecular arrangements formed in response to the stimulus such as those proposed by Garner (17), but it would be idle to attempt any precise molecular interpretation. The residual effects of speed on stretches may be referred to the elements which are not ruptured.

VI. *Correlation with Results of Gasser and Hill.*

It is relevant to this discussion to recall the results of Gasser and Hill on the effects of sudden stretches on frog's muscle. There, if the stretch was too great, the tension of the muscle was momentarily partially destroyed, but recovered again to its usual value. This suggests also a reparable rupture of certain muscle elements. On the other hand, in the case of Holothuria, where the phenomenon under discussion does not appear, sudden stretches (provided they do not actually tear the gross structure of the muscle) produce no such fall of tension (see Hill (19)). It is all the more reasonable to suppose, therefore, that the anomaly in the case of tortoise and crab is due to breaking or slipping of a part of the contractile elements and not to any fundamental difference of structure between them and dog-fish. We regard this structure as of the same type as the Model II.

The case for this view of muscle which we have been advocating is further strengthened by a more extended consideration of the results of Gasser and Hill. In the first place it is of interest to observe that, quite apart from any mechanical interpretation, our own results are in close accord with theirs. It is possible, indeed, without hypothesis to deduce one set of observations from the other. For example, if we accept the results of Gasser and Hill on the effects of a quick release, we can predict, for a muscle shortening at finite speed, tension-length curves of the same form as those we have actually observed. Let us assume that the continuous process of shortening is the limiting case of a discontinuous process consisting of a large number (n) of quick releases, Δy, occurring at times $\theta_1, \theta_2, \ldots, \theta_n$ after the beginning of movement, the limit being approached as the value of n becomes infinite. We now apply the results of Gasser and Hill to each quick release. They have shown that a release of extent Δy is followed by a drop of tension $\Delta F = k\ \Delta y / y_0$, where k is a constant and y_0 is the resting length of the muscle, and that this drop is followed by a recovery along a curve like that for the development of tension in an isometric contraction. This curve is well known to be approximately exponential in character, so that we may write for the force F exerted by the muscle at any time t after a single quick release Δy

$$\mathrm{F} = \mathrm{F}_0 - \Delta \mathrm{F} e^{-at} = \mathrm{F}_0 - \frac{k}{y_0} \Delta y e^{-at}.$$

Here F_0 is the equilibrium value of the tension for the new length and a is a constant. We now consider a discontinuous process of shortening consisting of a large number of quick releases. We suppose for simplicity that F_0 has the same value for all lengths covered (which is approximately the case for the range considered). Then at any time t after the first quick release the tension exerted by the muscle will be

$$\mathrm{F} = \mathrm{F}_0 - \frac{k}{y_0} \sum_{h=1}^{n} \Delta y_h e^{-a(t-\theta_h)}.$$

As n becomes infinite, corresponding to the case of the continuous shortening carried out in our experiments, this expression becomes

$$\mathrm{F} = \mathrm{F}_0 - \frac{k}{y_0} \int_{\theta=0}^{t} e^{-a(t-\theta)} \, dy/d\theta \, . \, d\theta.$$

If, as in our experiments, the shortening proceeds at constant speed

$$v = \frac{y - y_0}{\theta},$$

the value of this integral is

$$\mathrm{F} = \mathrm{F}_0 - \frac{kv}{y_0 a} \left(1 - e^{-\frac{a}{v}(y-y_0)}\right),$$

i.e., precisely the expression (equation (6)) which we have shown to describe the behaviour of muscle in our experiments in the case of shortening. The same expression might likewise be deduced for stretches in cases like dog-fish or Holothuria, where the complicating effects of breaking or slipping do not occur.

Now that we have shown the fundamental identity of our own and the results of Gasser and Hill, it is of interest to observe that the latter agree very closely with the mechanical interpretation we have given the former. In the first place the fact that a quick release of extent not greater than 15 per cent. of the resting length of the muscle leads not to the abolition, but only to a drop, of tension roughly proportional to the amount of shortening, shows that muscle cannot be a system like Model I, containing no element of free elasticity. In such a system any quick release, however small, leads to a temporary total disappearance of tension. On the other hand, this result is quite in accord with the behaviour of a system like Model II. If Model II, at equilibrium under a certain tension, be suddenly released, the top spring, undamped, will alone shorten ; the lower spring will be unable immediately to change its length on account of the damping vane. In this way equilibrium between the two springs will be destroyed ; the top spring will shorten by an amount greater than corresponds to equilibrium at the new length, and the tension exerted by the system, though not zero, will be less than the normal value for this length. At the same time a force will be brought to bear on the vane, causing it to move downward, so as to stretch the top spring and allow the lower spring to shorten until the new equilibrium is reached. The rate of this movement, which is the rate of attainment of the final tension for the new length, is proportional to the difference in tension between the two springs. From this it is clear that recovery from the initial drop must take place along an exponential curve. It may, in fact, be deduced from the equations describing the motion of the system that the tension F exerted at any time t after a sudden change of length (stretch or release) of extent Δy is given by the expression

$$\mathrm{F} = \mathrm{F}_0 + \frac{\alpha_2{}^2/l_2{}^2}{\alpha_1/l_1 + \alpha_2/l_2}\,\Delta y e^{-\frac{(\alpha_1/l_1 + \alpha_2/l_2)}{\eta}t},$$

where F_0 is the equilibrium tension for the new length. If the release be of such an extent that the top spring shortens to its unstretched length, we have a temporary disappearance of tension, just as in the case of muscle when the shortening is more than 15 per cent. of the resting length.

These results, therefore, of Gasser and Hill and of Hill confirm, both directly of themselves and indirectly by implying our own results, the amended viscous-elastic interpretation we have given to muscle.

In accordance with this interpretation it would, in fact, be possible to work out the absolute values of the three constants α_1/l_1, α_2/l_2, and η, for any given muscle, from its tension-length and work-speed curves. We have not actually carried out any such calculations, and it is doubtful of just how much value they would be, for it is obvious from the meanings we have assigned to α_1, α_2, and η that they depend on the size and shape as well as on the intrinsic character of the muscle. In order to calculate significant figures in this way it would be necessary to reduce them to values for 1 c.c. of muscle tissue referred to an axis parallel to the direction of the fibres.

VII. *A Theoretical Derivation of the Contraction Curve.*

A further point of interest arises in connection with the view advanced by Gasser and Hill that the development of tension in the isometric contraction is governed by the same factors as the redevelopment of tension after a sudden release. The view is based on the close similarity of the tension-time curves for both processes. This is precisely what would be expected if it were assumed that the muscle consists of two sets of elastic elements, one free and the other damped, the damped elements being the contractile part of the structure. In this case stimulation may be supposed to bring about a sudden change in the elastic properties of the contractile structures, which in consequence begin to shorten and stretch the purely elastic elements. Shortening of the contractile parts falls off logarithmically as the system approaches its new equilibrium, so that the change of tension with time will be the same as during recovery after a sudden release. The process of contraction could be simulated with the model we have been discussing by giving the lower spring a sudden extension without allowing the vessel containing the damping liquid to move. It is obvious that the effect of such a stretch would be strictly analogous to the effect of a sudden release of the upper spring. A similar comparison might be made with the effects of a sudden stretch. It is plain that quite a different result would follow if it were the free elastic elements which were responsible for contraction. In this case there would be a *sudden* initial rise of tension at the moment of contraction, followed by a drop to the equilibrium value, quite the contrary of what is actually observed.

Not only does this view explain the fundamental similarity of an isometric contraction and a redevelopment of tension after a release, but it enables us to see why a sudden shortening of 15 per cent. should lead to an abolition of all tension in the muscle. For this purpose we have only to suppose that when the isometrically contracting muscle attains its maximum tension, the free elastic elements are stretched by an amount equal to 15 per cent. of the length of the muscle.

These free elastic elements may indeed be regarded as "buffers." They protect the muscle in case of sudden changes of length. If the muscle were a simple viscous-elastic system like Model I, any sudden extension such as might occur in the case of certain movements of the animal would produce a tremendous immediate rise of tension which would result in shock if not in actual rupture of the muscle. On the other hand, the viscous character of the contractile elements serves to prevent too sudden changes of tension when the muscle contracts isometrically or when it shortens : great strains would be introduced by the immediate development of maximum tension. Here, again, the elastic buffers play their rôle, for without them any rapid shortening of small extent might lead to an immediate disappearance of tension, and the efficiency of the muscle would be greatly reduced. We seem, therefore, to be dealing with a highly effective and adaptive arrangement.

It might be maintained that an objection to this point of view lies in the fact that in reality the curves for the isometric development of tension are not strictly logarithmic but contain a point of inflection near the start. It is, however, very simple to explain this apparent inconsistency as due to finite time (small, to be sure, when compared with the time required for the development of maximal tension) occupied by the chemical events determining the change in the elastic properties, and in general in the colloidal state, of the contractile elements. Certainly this change cannot occur instantaneously, even though Gasser and Hill have shown, by suitably timed stretches, that there is a great rise in viscosity before there is any appreciable development of tension on the part of the muscle in response to stimulation.

It is important to remark here that in the foregoing analysis we have made use of concrete models simply as illustration of *types* of mechanical systems and to facilitate the deduction of the mathematical description of the behaviour of these systems. We do not in the least suggest any actual resemblance between the muscle and these arbitrary structures. It might have been supposed that certain histological elements in the structure of the muscle were the counterparts of the two different elastic components of Model II, *e.g.*, the isotropic and the anisotropic bands in the fibres might be indentified with the free and damped elastic elements of the model. The fact that both smooth and striped muscle display similar behaviour makes any such procedure very dangerous. It is far more likely that the behaviour of the muscle is correlated with a much finer, molecular structure.

VIII. *Effect of Fatigue.*

Some experiments also have been performed with a view to investigate the effects of fatigue on viscosity. They are of importance in the question of the relation of the viscosity to the contraction. It is a well-known fact that as a muscle becomes fatigued the speed of its response to stimulation is decreased, *i.e.*, the time taken to develop maximal tension isometrically is increased. This may be explained in accordance with our view as due to an increase in the viscosity of the contractile elements, owing possibly to the accumulation in the muscle of some breakdown products of contraction. This explanation implies, however, a corresponding increase with fatigue in the "viscous" loss of work when the muscle shortens at a given speed, for we have shown above that both phenomena—the development of tension along an exponential course and the loss of work for any shortening at finite speed—can be ascribed to the same cause, namely, viscosity, or its analogue, in muscle. In order to test this point we therefore carried with dog-fish muscle a large number of releases at a given speed until fatigue was well established. When we measured the ratio of the " viscous " loss to the ideal work at zero speed in successive shortenings at a given speed, we found a large increase (from 47 to 77 per cent.) between the first and the last observations. The actual experimental results are shown in fig. 11. This all provides additional evidence (*a*) that the factor determining the rate of the initial development of tension isometrically is the same as that governing the irreversible loss of work in any shortening at finite speed, viz., " viscosity," and (*b*) that fatigue is intimately connected with an increase of the " viscosity " of the contractile elements.

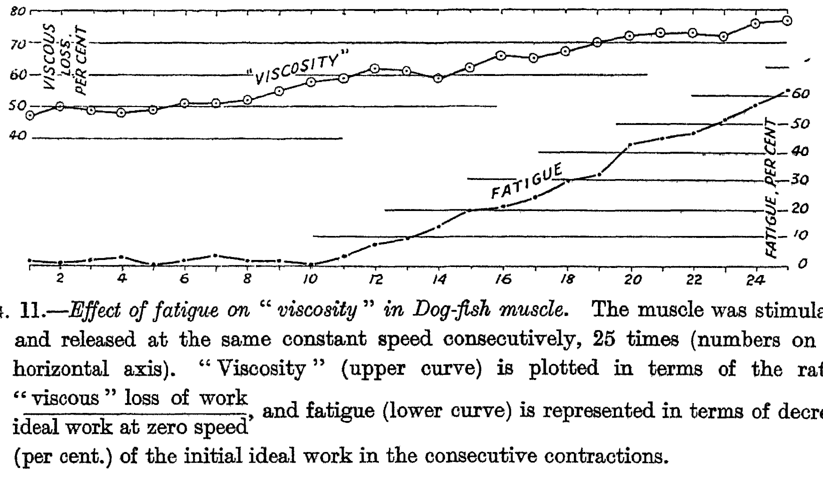

FIG. 11.—*Effect of fatigue on " viscosity " in Dog-fish muscle.* The muscle was stimulated and released at the same constant speed consecutively, 25 times (numbers on the horizontal axis). " Viscosity " (upper curve) is plotted in terms of the ratio: $\frac{\text{“viscous” loss of work}}{\text{ideal work at zero speed}}$, and fatigue (lower curve) is represented in terms of decrease (per cent.) of the initial ideal work in the consecutive contractions.

IX. *Conclusion.*

We referred above briefly to a proposed molecular picture of the type of structure involved in muscular contraction. Garner has suggested that contraction may be due to a rearrangement of the molecular pattern of lipoid molecules oriented in an anisotropic film on the surface of the contractile elements. Recent work on the area covered by the lipoid substances contained in muscle makes it probable that the elements in question are fibrils rather than fibres. But in any case it is of considerable interest in this connection to observe the analogy between the hysteresis curves obtained by Adam with contracting and expanding films and the results obtained with muscle (see Hill (16), p. 105). The curve shown in fig. 4 of Hill's paper, taken from the paper of Adam and Dyer (18), is quite similar to the tension-length curves for muscle with ordinates and abscissæ interchanged. It must, of course, be remembered Adam's films show pressures not tensions. The films tend to expand, not contract. Certainly, however, the analogy is suggestive.

But whatever be the intimate structure postulated for muscle, the facts and arguments presented in this paper make it very probable that we are dealing with a system which is, mechanically considered, of the type represented by Model II. Muscle behaves, in fact, with a few easily explained deviations, precisely like this model; and it is reasonable to suppose that its structure contains viscous and elastic elements which are the counterparts of those of the model, the contractile elements being probably those showing the property of viscosity.

X. *Summary.*

(1) By means of an improved form of myograph very accurate tension-length curves were obtained for stretches and releases carried out on various types of muscles, smooth and striated, at any desired constant speed. A description is given of the characteristics of these curves, as well as of others derived from them, showing the relation of external work done to the speed of stretch or release.

(2) These curves confirm the observations of Fick and of Hill that tension developed in isometric contraction is reduced by release and increased by stretch and that the effect increases with the speed. They also confirm the views, advanced by Hill, that this effect is caused by "viscosity." At the same time the fact contained in our curves, that tension never falls or rises instantaneously at the moment when release or stretch commences, suggests the presence of free elasticity undamped by "viscosity."

(3) A mechanical model was constructed to investigate this (fig. 4 (II)) in which the three factors concerned were represented separately, namely, damped elasticity, viscosity producing the damping, and free undamped elasticity. A mathematical treatment of this model is given and a careful analysis of the behaviour of the muscle in the light of this model leads to the conclusion that muscle is, mechanically considered, a system of this type.

(4) Our results suggest the following explanation of the phenomena (see figs. 2 and 10). When the muscle is stimulated isometrically, tension is set up in it, which is shared by the viscous-elastic and by the purely elastic elements. When the muscle is then released (or stretched) at a constant speed, the viscous-elastic elements lag behind and only the purely elastic elements contract (or are stretched) at the first instant, the presence of the latter preventing the tension from dropping (rising) instantaneously; the tension-length curve, therefore, starts at an angle which is determined solely by the elastic coefficient

of the purely elastic elements and is the same for all speeds of the release or stretch. Subsequently the viscous-elastic elements also gradually shorten (lengthen), thereby diverting the curve from the initial direction along an exponential course. Finally a steady state of lag is reached and the tension remains reduced (increased) throughout the movement by a constant amount, which is greater with greater speeds. In other words, the curves for all speeds tend to become parallel to each other and to the line of infinitely slow release or stretch. When the release (stretch) stops, the viscous-elastic elements continue to shorten (lengthen), stretching (being stretched by) the purely elastic elements until the equilibrium tension is reached.

(5) The exponential form of the isometric tension-time curve showing the initial development of tension on stimulation and its similarity to the curve of redevelopment after release (Gasser and Hill) are easily explained on the basis of our theory. We have only to assume that the viscous-elastic elements are the actual contractile structures and that they change their elastic properties suddenly on stimulation and start thereupon to shorten and to stretch the elastic elements until a new equilibrium is reached along an exponential curve.

(6) The purely elastic elements may be regarded as " buffers," which take up any sudden stresses produced by the contraction of the viscous elements, or by quick movements of the animal, and which also prevent an immediate disappearance of tension when the muscle rapidly shortens by a small amount.

(7) In the model, the tension is as much reduced by a release as it is raised by a stretch at the same speed, provided (*a*) that no buckling occurs at quicker releases, so that negative tension (compression) is also transmitted, and (*b*) that no slipping or breaking occurs at quicker stretches when the tension is raised beyond a certain limit. In some muscles the tension drops less for releases than it rises for stretches, suggesting buckling of some of the slowest fibres. In some other muscles, on the contrary, the rise of tension in stretches is less than corresponds to the drop in releases, the tension appearing to be cut short at a certain limit, suggesting slipping or breaking (reparable) of some of the elements of the active muscle.

(8) Fatigue has the effect of increasing the " viscosity " as well as of decreasing the tension.

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The Action of Glucosone on Normal Animals (Mice) and its possible Significance in Metabolism.

By Alexander Hynd.

(Communicated by Principal Sir James Irvine, F.R.S.—Received January 26, 1927.)

(From the Physiology Department, University of St. Andrews.)

Introduction.

Reactive Forms of Glucose.

The almost simultaneous discovery by Emil Fischer (1) in Berlin and by Irvine, Fyfe and Hogg (2) at St. Andrews of the existence of reactive forms of sugars, coupled with the observations of Hewitt and Pryde (3) on the optical changes undergone by a sugar solution when introduced into a loop of living intestine, initiated the speculations of Winter and Smith (4, 5, 6) on the part played by the so-called "*γ-glucose*" in carbohydrate metabolism. The latter workers found that the optical activity and the copper-reducing power of a protein-free filtrate from normal blood were not compatible with the idea that the sugar present existed entirely as the ordinary equilibrium mixture of αβ-glucose. Having also observed that the fresh filtrate reduced potassium permanganate solution in the cold, they concluded that the glucose of normal blood exists in the unstable γ-form. Further, as the examination of the blood of diabetics pointed to their blood sugar being present as αβ-glucose, they suggested that the conversion of the relatively stable αβ-variety into the reactive γ-form was an essential step for utilisation of the sugar by the body cells and that this conversion was brought about by the action of insulin.

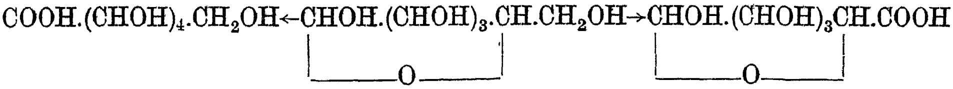
These speculations unfortunately have not stood the test of later experiments (7, 8, 9), and though in 1923 Irvine (10), in the course of a review of the chemical evidence for the existence of a γ-form of glucose, pointed out the possibility that such a form may be produced in the body as an intermediary product during the glucose metabolism, he, in collaboration with Herring and Macleod (11), soon afterwards furnished experimental evidence against the theory of Winter and Smith, by showing that glucose monoacetone, a readily hydrolysable derivative of γ-glucose, is without action when administered in cases of insulin hypoglycæmia. More recently, however, the same idea, or one very similar, has been revived by Lundsgaard and Holbøll (12, 13). They claim that when insulin and muscle tissue are added to a solution of ordinary αβ-glucose, with a specific rotation of +52·5°, and the mixture incubated at 37° C., no change in reducing power takes place, but the optical rotatory value decreases to a new value varying from +15·9° to +42·2°, because of the formation of what they call "new" or "neo-glucose." Insulin alone, or muscle tissue alone, does not produce the change, but the two factors, insulin and the active substance or principle in fresh finely minced muscle tissue, must act simultaneously (14).

Ahlgren (15) proposed the term "glukomutin" for the active substance in muscle, but Lundsgaard and Holbøll prefer to call it "insulin complement," which, in their most recent paper (16), they describe as not identical with the muscle co-ferment of Meyerhof (17). They claim to have demonstrated that "neo-glucose" is present in normal blood, and also, to a somewhat greater extent, in various biological fluids (spinal, œdema, and pleural), but is absent from the blood of diabetics (18, 19), so that they regard "neo-glucose" to be an essential link in the transformation of glucose which takes place in the animal organism. Already, however, many workers, including Barbour (20), Paul (21), Anderson and Carruthers (22), Harris, Lasker, and Ringer (23), Beard and Jersey (24), and Iwasaki (25), though following closely the technique of the Danish investigators, have been unable to confirm their results as to the production of "neo-glucose" *in vitro* from glucose solutions, to which insulin and fresh muscle have been added.

In view of the nature of the work now to be described, which indicates that special importance should be attached to the compound known as *glucosone*, it appears desirable to review briefly the first oxidation products of glucose.

Oxidation Products of Glucose.

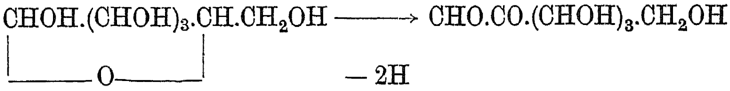
Despite the prominence given in recent years to reactive forms of glucose, as detailed in the preceding paragraph, the present writer has for a long time held the view that in carbohydrate metabolism some oxidation product of glucose must play an important rôle. Yet the two best-known first-oxidation products, namely, gluconic acid and glycuronic acid, have been shown to play no part in carbohydrate metabolism, and indeed are of no physiological significance apart from the indirect use of glycuronic acid as a detoxicating agent. These derivatives are formed from glucose by oxidation at one or other of the terminal carbon atoms of the sugar chain, attack at the



$$\mathrm{COOH.(CHOH)_4.CH_2OH \leftarrow \underbrace{CHOH.(CHOH)_3.CH}_{O}.CH_2OH \rightarrow \underbrace{CHOH.(CHOH)_3CH}_{O}.COOH}$$

$C_6H_{11}O_7$	$C_6H_{12}O_6$	$C_6H_{10}O_7$
(*d.* gluconic acid).	(*d.* glucose).	(*d.* glycuronic acid).

reducing group giving gluconic acid and attack at the primary alcoholic group giving glycuronic acid. Both compounds, it will be noticed, contain one atom of oxygen more than the original glucose. But there exists another type of oxidation product, which contains the same number of oxygen atoms but fewer hydrogen atoms than glucose, namely—glucosone, the formation of which may be represented by the following equation:—



$$\underbrace{\mathrm{CHOH.(CHOH)_3.CH}}_{\mathrm{O}}\mathrm{.CH_2OH} \xrightarrow[-2\mathrm{H}]{} \mathrm{CHO.CO.(CHOH)_3.CH_2OH}$$

(*d.* glucose). (*d.* glucosone).

In this paper, Fischer's formula for glucosone is retained for simplicity, although it is possible that the compound may, at some future date, be proved to contain in its molecule one or perhaps two oxidic rings.

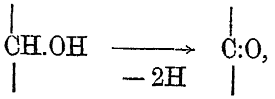
Glucosone.

Though glucosone is well known to chemists as an intermediate stage in Fischer's brilliant work on the synthesis of the sugars (26), its chemical properties and reactions have not been fully examined and its physiological behaviour has been more or less neglected. The only reference to the latter, which the author has been able to find, is the experiment of Levene and Meyer (27), who report that they could find no evidence that glucosone is acted upon by tissues. Notwithstanding this result, the keto-aldehydic structure of the compound and its relationship to glucose suggested to the author that further study might prove glucosone to be of considerable importance, both physiologically and also as a starting material for the synthesis of certain carbohydrate complexes.

The difficulties encountered in the preparation of glucosone in sufficient quantity and of reliable purity have delayed both these types of investigation, and as, in the meantime, Thannhauser and Jenke (28) have shown that the compound is utilised by diabetics, a preliminary communication on the effect of glucosone on normal mice is presented without further delay.

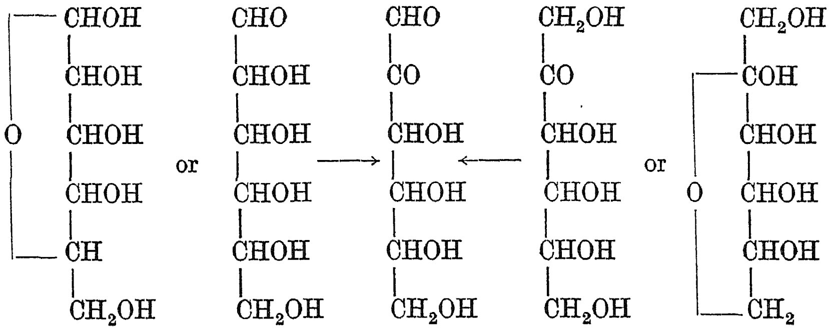
Nomenclature.

As already indicated, glucosone is an oxidation product of glucose, and, as a consequence, it is sometimes referred to as "oxyglucose." The latter term, however, though perfectly correct, suggests the presence of an extra OH group, whereas the change from glucose involves oxidation of the secondary alcoholic group, adjacent to the reducing group, into a ket*one* group, so that the term



$$\mathrm{\overset{|}{\underset{|}{C}}H.OH} \xrightarrow[-2\mathrm{H}]{} \mathrm{\overset{|}{\underset{|}{C}}:O},$$

"glucos*one*" is preferred, although it must be admitted that its use may conceivably lead to confusion with gluc*osazone*, and also with gluc*osan*. Perhaps a less misleading term would be "aldo-fructose," which would be equally correct, as the compound in question is, at one and the same time, the keto-derivative of glucose and the aldo-derivative of fructose :—



(glucose) (glucosone or aldofructose). (fructose).

PREPARATION OF GLUCOSONE.

(1) *Direct Oxidation of Glucose or Fructose.*

Hydrogen peroxide in the presence of a ferrous salt (Fenton's reagent) is the oxidising agent usually employed to accomplish the conversion of either glucose or fructose into glucosone. As this method has been applied to both the above-mentioned sugars by Morrell and Crofts (29) with evidence of the formation of a considerable amount of glucosone, the process was tried on several occasions, but, as numerous other oxidation products are formed simultaneously, it was found impossible to isolate the desired compound free from other substances. On this account, material prepared by the use of hydrogen peroxide has not been utilised in the animal experiments subsequently described in this paper.

(2) *Fischer's Method.*

The method originally described by Fischer (30, 31) was accordingly employed for the preparation, even though it involves the use of reagents which are better avoided in the preparation of material intended for physiological experiment. The method consists in preparing from glucose or fructose, glucose phenylosazone by the interaction of phenylhydrazine, which process, it should be noted, involves an oxidation. The subsequent procedure, by means of which glucose phenylosazone is converted into glucosone, followed precisely the lines prescribed by Fischer (*loc. cit.*), until a syrupy product was obtained, free from nitrogen, but still containing mineral matter. To free the product from every trace of inorganic material, extraction with absolute alcohol and subsequent concentration to a syrup were repeated several (at least four) times. The resulting glucosone was a clear, stiff, almost colourless syrup, which reacted only very slightly acid to litmus paper and left no residue on ignition. It was also proved to be free from nitrogen. The yield of purified product was poor, varying from 16 to 30 per cent. of the theoretical amount. So far, the compound has not been obtained crystalline, and accordingly it is difficult to determine the purity of any particular sample. Though glucosone may be regarded as a fructose derivative, it does not give Seliwanoff's test, but the compound reduces Fehling's solution in the cold and reacts with phenylhydrazine readily at 30°—40° C. The compound is not fermentable by yeast and is slightly lævorotatory (average value for specific rotation $= - 3 \cdot 25^\circ$). As no change in rotation was detected in an approximately 8 per cent. aqueous solution of the compound after standing for 10 weeks at laboratory temperature, it is concluded that glucosone is comparatively stable *in vitro*, provided the reaction of the solution is not alkaline.

(3) *Application of Herzfeld's Method.*

Herzfeld's process (32), depending on the decomposition of osazones by means of benzaldehyde, was applied by Fischer and Armstrong (33) for the preparation of the osones of disaccharides. Though Morrell and Crofts (34) refer to unsuccessful attempts to apply this method to glucose phenylosazone, a modification has now been worked out for the preparation of glucosone by this process. Though this method has certain advantages, in that no inorganic materials are introduced in the process, the yields so far obtained have not been satisfactory, and further experimentation will be necessary. The resulting product, however, was in every respect identical with that prepared by Fischer's process and displayed the same physiological properties.

(4) *Hydrolysis of the Osones from* (*a*) *Maltose and* (*b*) *Lactose.*

Maltosone and lactosone were also prepared by the Fischer and Armstrong method (*loc. cit.*). Calculated on the weight of the phenylosazones employed, very good yields of these two compounds were obtained.

As acid decomposes maltosone with formation of glucosone and glucose, while lactosone on similar treatment gives glucosone and galactose, the hydrolysis of these two compounds was carried out. This was effected readily by boiling with dilute sulphuric acid, and the formation of glucosone in each case was confirmed by its physiological action, as will be described later.

Analysis of Glucosone.

It may be advisable at this point to emphasise the fact that, as oxidation products of sugars are notoriously liable to be contaminated with by-products, every endeavour was made to prepare glucosone in the purest condition possible. Evidence that this was accomplished with a considerable degree of success is obtained from the following facts.

Although four distinct methods have just been outlined in this paper for the production of glucosone, the product in each case has been found to display identical behaviour when injected into the animal organism.

The specific rotatory power of the various preparations of purified glucosone also showed little variation. The minimum value recorded was $-2{\cdot}94^\circ$, and the maximum $-3{\cdot}68^\circ$, with a mean of $-3{\cdot}25^\circ$, so that the variation is only very slight, when consideration is taken of the low optical activity displayed by the compound.

Further, by heating a known volume ot a standard solution of glucosone with excess of phenylhydrazine at 37° C., and collecting, on a tared Gooch crucible, the glucose phenylosazone which had formed, an estimate of the strength of a solution of the compound can be arrived at. Thus 2 c.c. of an approximately 4 per cent. solution of glucosone gave 0·1515 gm. glucose phenylosazone; hence percentage glucosone $= 0{\cdot}1515 \times \frac{178}{358} \times \frac{100}{2} = 3{\cdot}765$ per cent.

Then to 0·2 c.c. of the same solution, 15 c.c. $0{\cdot}1929 \frac{N}{1}$ $KMnO_4$ and 25 c.c. 10 per cent. KOH were added, and the mixture boiled over a micro-burner for ten minutes to bring about complete oxidation. Thereafter 25 c.c. of 25 per cent. H_2SO_4 and 10 c.c. $0{\cdot}2438 \frac{N}{1}$ oxalic acid were added to the hot solution, which then required 2·48 c.c. of the standard $KMnO_4$ solution for back titration. As 1 c.c. $\frac{N}{1}$ $KMnO_4$ is required for the complete oxidation of 8·17 mg, glucosone, the following calculation gives the percentage of glucosone in the solution.

Amount of $KMnO_4$ solution required for oxidation =

Total $KMnO_4$ solution used — Equivalent of 10 c.c. oxalic acid solution, $= (15 + 2{\cdot}48 - 12{\cdot}64)$ c.c. $= 4{\cdot}84$ c.c. $KMnO_4$.

$$\text{Therefore, percentage glucosone} = 4 \cdot 84 \times 0 \cdot 1929 \times 8 \cdot 17 \times \frac{100}{2} \times \frac{1}{1000} \text{ per cent.}$$
$$= 3 \cdot 814 \text{ per cent.}$$

The close agreement between the percentage of glucosone, estimated from the weight of resulting glucose phenylosazone (3·765 per cent.), and that determined from the complete oxidation of the compound by potassium permanganate (3·814 per cent.), warrants the assumption that the material employed was essentially pure glucosone.

It is to be noted that only material of this quality was employed in studying the physiological behaviour of the compound, and as only a small amount of such material was available mice were selected as the most suitable animals for the experiments.

PHYSIOLOGICAL ACTION.

(1) *The Action of Glucosone on Insulinised Mice.*

The first experiments were undertaken to ascertain whether glucosone alleviates the symptoms caused by insulin. A number of mice were injected with a suitable dose of insulin, and after severe symptoms accompanied by convulsions had developed, glucosone was administered subcutaneously. Only on one occasion out of ten experiments did the injection of glucosone relieve the condition—the usual effect being to intensify the symptoms and to cause death of the animal in five to ten minutes, even though glucose was also injected.

(2) *The Action of Glucosone on Normal Mice.*

Normal mice were injected with a dose, proportional to their body weight, of the same glucosone as was used in the experiments with insulinised mice. In the course of five to seven minutes the development of definite symptoms was noticed, and after about 20 minutes the animals presented a condition very *similar to that produced by an injection of insulin.* The ears were perked up, the eyes bulging, the tail held erect, and the hind limbs sprawling. The animals became extremely irritable, and convulsive movements were frequently noticed. Sometimes squeaking was a marked symptom, and an almost constant feature shown by the injected animals was a tendency to walk backwards with a simultaneous rotatory movement. Drowsiness developed later, accompanied by weakness of both the fore- and hind-limbs. This condition has been produced invariably (28 distinct experiments).

In the majority of those cases in which the symptoms were marked and actual convulsions ensued, death in coma followed. With larger dosage, the symptoms developed more quickly and were more severe; death usually occurred within 10 minutes after the injection. With a smaller dose, the symptoms naturally were less marked, and the animals recovered completely and permanently if left to themselves.

There appears to be a very narrow margin between the minimum lethal dose and the non-lethal dose. Thus it was found that, in the case of mice, 2·4 mg. per gram of body weight is non-lethal, but an additional 0·2 mg. produces a fatal result. A concentrated solution seemed to be more effective than the same amount of glucosone given in a more dilute solution.

It may be mentioned that the eyes of mice which die as the result of an overdose of glucosone remain in a state of extreme exophthalmos, and after 20 to 30 minutes exhibit a peculiar whitening (probably of the corneal epithelium). This same effect has also been produced in a mouse (21 gm.) by the injection of a large dose of insulin ($1\frac{1}{2}$ units), followed, as soon as symptoms appeared, by a large injection of glucose (10 minims of a 15 per cent. solution).

(3) *The Action of Maltosone on Normal Mice.*

A mouse, 22 gm., was given subcutaneously 14 minims of a maltosone solution, which analysis showed to be 11·66 per cent. The dose, therefore, contained an amount of maltosone equivalent to a lethal dose of glucosone. No symptoms resulted from the injection; the animal remained perfectly normal.

A mouse, weighing 23 gm., was similarly injected with 13·5 minims of an approximately 23·32 per cent. solution, of maltosone, but again no effect was noticed, though twice the equivalent of glucosone was used in this experiment.

These experiments have been repeated on four distinct occasions with identical results.

Further, 5 c.c. of the 23·32 per cent. solution of maltosone were diluted with 12 c.c. of water and 3 c.c. of 25 per cent. H_2SO_4 added. The mixture was then boiled gently under a reflux condenser for one hour. The brownish coloured solution after dilution was freed from acid by shaking with a slight excess of barium carbonate, filtered from the barium residues, and then decolorised with charcoal. The colourless filtrate was concentrated *in vacuo* to a syrup, which was then extracted with hot absolute alcohol. The filtered alcoholic extract was evaporated under diminished pressure, and the residual syrup dissolved in a little water. The volume was finally adjusted to 5 c.c., so that, assuming no loss and complete hydrolysis of the maltosone, the resulting solution would contain, in addition to glucose, about 0·58 gm. of glucosone.

A mouse, 19 gm., was injected with 7 minims of the glucosone solution thus obtained. Two minutes afterwards, violent squeaking resulted, and severe symptoms soon followed. In 12 minutes the mouse was comatose, and died 15 minutes after the injection.

(4) *The Action of Lactosone on Normal Mice.*

A 13·93 per cent. solution of lactosone was used. A mouse, 25 gm., was injected with 13 minims of this solution, but no effect was produced. Then 13 minims of an approximately 27·86 per cent. solution of lactosone were given to a mouse weighing 25·5 gm. No symptoms ensued.

Five c.c. of the 27·86 per cent. solution of lactosone were then hydrolysed as described above for maltosone, and a solution obtained containing about 0·7 gm. glucosone in 5 c.c.

Four minutes after the injection of 6 minims of this solution into a mouse weighing 22·5 gm., the animal showed severe symptoms and squeaked continuously. A violent convulsion occurred five minutes later; the mouse lay on its side helpless and died about 10 minutes after the injection.

(5) *The Influence of Glucose on the Action of Glucosone.*

The amount of glucosone given in each of the following experiments was sufficient to produce severe symptoms in a normal mouse, being just under the lethal dose.

The injection of glucose after the full development of the symptoms due to a dose of glucosone, resulted in *two* cases, in the slight recovery of the animals, followed by relapse and death, and in *one* case, of slow but apparently complete recovery, followed by sudden death.

In *three* experiments, when the glucose and glucosone were injected simultaneously, the glucosone symptoms were diminished in all three cases, one animal recovering rapidly, the other two more slowly, and one of the latter being, next morning, found dead with signs of hæmorrhage at the nose.

In *six* experiments glucose was injected about five or six minutes before the glucosone. In all these the symptoms noted were both delayed in their development and diminished in intensity, as compared with control animals receiving the same dose of glucosone but no glucose.

(6) *The Influence of Adrenaline on the Action of Glucosone.*

Two sets of experiments were carried out with adrenaline: the amount of glucosone used in each experiment was just short of the lethal dose.

(*a*) Two mice, 16 gm. each, were injected with 35 mg. glucosone, and 15 minutes later, at which time both animals were moribund, a dose of 1 in 2000 adrenaline (Parke Davis) was given to each subcutaneously. Ten minutes afterwards both had recovered sufficiently to run about, but the animals were not normal and looked like typical "insulinised" mice, except that the body temperature was raised. Five minutes later, convulsions ensued, followed by improvement. One animal had a severe convulsion twelve minutes afterwards and died (blood-stained froth from mouth). The other mouse apparently recovered, but was found dead next morning.

(*b*) Adrenaline (4 minims of 1 in 4000) was injected into *six* mice, weighing from 15 to 25 gm., immediately before the injection of glucosone. In every case, the symptoms which ensued differed from those produced by glucosone alone. Squeaking was noted in nearly all cases: the body temperature was raised: the animals were very excited and lay in a characteristic manner—stretched out with the nose extended over the fore-paws. There was no protuberance of the eyeballs, but the eyes were kept wide open. All these animals recovered without any further treatment.

(7) *The Influence of Pituitrin on the Action of Glucosone.*

Two sets of experiments were also carried out with pituitrin (Parke Davis). The dose of glucosone used in each case was again just short of the lethal amount.

(*a*) *Two* mice, 13·6 gm. and 14·5 gm. respectively, were rendered comatose by the dose of glucosone in 11 and 7 minutes respectively. Each was then injected with 4 minims pituitrin. Seven minutes after receiving the pituitrin, both were able to walk about. The animals were very drowsy for about an hour afterwards, and though able to move about, they remained very still, sitting hunched up with ruffled fur and eyes half-closed.

(*b*) *Six* mice, varying from 15 gm. to 18 gm. in weight, were each given 4 minims pituitrin, and immediately afterwards injected with a proportionate sub-lethal dose of glucosone. Only in one case was there a development of marked symptoms, and these quickly passed off, the animal becoming drowsy like the others. All soon returned to the normal condition except for some lethargy from which they were easily aroused. There was no evidence of any marked increase or decrease of the body temperature.

(8) *The Influence of Acetoacetic Acid on the Action of Glucosone.*

For the following experiments a solution of the sodium salt of acetoacetic acid was prepared according to the directions given by Shaffer (35). *Eight* experiments were carried out with this material on mice of an average weight of 18·5 gm.

In one case, 2·8 minims of an approximately 20 per cent. solution of acetoacetic acid (Na) were injected after severe symptoms, due to a previous injection of glucosone, had developed. The animal remained practically moribund for half an hour, then the occurrence of winking movements of the eyelids was noticed, followed by definite signs of improvement and complete recovery in the course of an hour.

In other seven experiments, the acetoacetic acid was injected five minutes before the glucosone. In two of these no symptoms developed, while in the other five only very slight symptoms were detected about ten minutes after the glucosone injection, and these soon disappeared, the animals becoming perfectly normal, though in some cases the behaviour of the animals resembled that of the mice treated with adrenaline.

Two control experiments were carried out at the same time, namely :—(1) a mouse, which received the same dose of glucosone, showed in five minutes marked symptoms—bulging eyes, sprawling hind-legs, stiff tail, and, a little later, was lying comatose, but after two hours spontaneous recovery took place, and

(2) a mouse, which received the same dose of acetoacetic acid showed no symptoms.

(9) *Blood Sugar Determinations.*

The following estimations were carried out in order to provide data necessary for the discussion which follows.

(*a*) *Normal Mice.*—In connection with other work, a considerable number of blood sugar determinations of normal mice has been carried out by Maclean's method, the values varied from 0·124 to 0·188 per cent., with a mean percentage of 0·161 per cent. glucose. This is quoted for comparison.

(*b*) *Mice after an injection of Glucosone.*—Glucosone, 70 mg., was given to a 26·2 gm. mouse. Symptoms developed almost immediately, and in ten minutes the animal was moribund. Blood was then removed from the heart, and sugar estimations carried out. The mean value of three determinations was 0·246 per cent. glucose.

A similar experiment, using a 23·9 gm. mouse and injecting 64 mg. glucosone, gave 0·230 per cent. glucose (mean of two determinations).

(*c*) *Mouse after an Injection of Glucose.*—As a control experiment, 75 mg. of glucose were injected into a 29 gm. mouse, and the blood sugar determined 10 to 12 minutes afterwards. The value found was 0·320 per cent. glucose.

DISCUSSION.

Before entering on a discussion of the possible significance of the experimental results just recorded, it is necessary, in the first place, to emphasise the fact that the same findings were obtained, not only with several different samples of glucosone prepared by Fischer's process, but also with preparations by the modified Armstrong method. As in the latter neither lead nor barium had been employed, none of the symptoms observed can be attributed to the use of the carbonates of these metals in Fischer's process. Further, a control experiment with a very dilute solution of baryta (but which gave the tests for barium readily) was carried out with negative results, so that the conclusion was drawn that the symptoms produced on the injection of glucosone into a normal animal were due to the action of that compound alone and not to any by-product or impurity.

As to whether glucosone was simply a toxic compound, or a substance of physiological importance, some indirect but important evidence was obtained from experiments with the osones of the disaccharides, lactose and maltose. Neither of those compounds on injection produced the slightest effect. This result is of considerable significance. The non-activity of maltosone and of lactosone contrasted with the activity of glucosone when prepared by an analogous process, disposes of the possibility that the symptoms observed might be due to impurities. Further, as after hydrolysis, the solutions obtained from the decomposition of both lactosone and maltosone were found, on injection into mice, to be physiologically active, evidence of the specific action and physiological importance of glucosone was obtained.

It is suggested that glucosone produces the symptoms described, not merely because of its keto-aldehydic group, though this portion of the molecule is more than likely involved, but because the compound is an essential intermediate in the metabolism of carbohydrate. The same keto-aldehydic group is present in both lactosone and maltosone, yet both are quite inactive, so that the specific nature of the glucose derivative is of the utmost significance.

The striking similarity between the symptoms produced by the injection of glucosone and those following the administration of insulin to normal mice, as well as the fact that both sets of symptoms are modified by the action of either adrenaline or pituitrin, indicate that there is a close connection between glucosone and insulin. The recent experiments by Lesser and his co-workers (36), by Cori and Cori (37), and by Best, Dale, Hoet and Marks (38, 39), and also the clinical studies by Lawrence (40), have confirmed the opinion originally expressed by Dale (41) that the ultimate fate of the blood glucose is its conversion into glycogen or its complete oxidation to water and carbon dioxide. Nevertheless, an important part of the problem of the effect of insulin on carbohydrate metabolism remains to be elucidated, namely, the discovery of the intermediate stages. So far, apart from the reactive forms of glucose, referred to in the introduction, and unknown but presumed easily oxidised complexes, no sugar compound has been suggested as an intermediate except hexose diphosphate. Though numerous workers have supported the claims of this compound in this capacity, its exact rôle in carbohydrate metabolism is not yet clear, and Kolodziejska and Funk (42) have recently pointed out that insulin plays no significant part in the *total* phosphorus metabolism. The present writer, in seeking to obtain an explanation for the experimental results described in this communication, suggests that glucosone may be an intermediate metabolite, and that its formation, in the animal organism, arises from the action of insulin on the blood glucose, which is considered to be present, initially at least, in the ordinary $\alpha\beta$-modification.

According to this view the action of insulin would be regarded essentially as that of an oxidase, which converts the inert $\alpha\beta$-glucose, either directly or indirectly into glucosone, which compound, in an alkaline medium such as exists in the body cells, is then ultimately transformed into glycogen, and also into various other products according to the immediate requirements of the organism. The acceptance of this view gives a satisfactory explanation of the definite increase in the oxygen consumption observed by Burn and Dale (43) in the initial stages of the action of insulin, and is, in other respects, consistent with our present knowledge of the action of the pancreatic hormone.

The marked difference in the effect of glucose on the glucosone symptoms and on those of an "insulin hypoglycæmia," might seem to be opposed to the above hypothesis, but it is readily explained by the fact that in the former case there is *probably* no diminution of blood sugar (glucose). Comparison of the blood sugar value of a normal mouse (0·161 per cent.) with that existing when an injection of a fairly large dose of glucosone had produced its maximum effect (0·238 per cent.), indicates that the blood has a high reducing power even when the symptoms are most severe. One is not quite justified, however, in concluding that the glucose content is high, for it is to be remembered: (1) that the blood was examined 15 minutes after the injection of glucosone; and (2) that glucosone is itself a reducing substance exerting on Maclean's copper solution 30–40 per cent. of the reducing power of glucose. The high values obtained are, therefore, due probably to the presence of glucosone and the actual *glucose* content would not be far removed from the normal. Accordingly, until further work can be carried out, it is assumed that the symptoms produced by an injection of glucosone are not due to a "glucopenia."

This raises the question as to what the toxic phenomena observed after insulin are due. It is well known that a low blood sugar may exist without being accompanied by convulsions. Also, as Laqueur and Grevenstuck (44) point out, it is somewhat paradoxical that a large dose of insulin will sometimes not induce a convulsion, while a smaller dose will, although the blood sugar content is greatly reduced in both cases. Evidently "insulin hypoglycæmia" covers a whole series of symptoms, and only some of them arise as the result of a low blood glucose content. This agrees with the observation that the effect of glucosone is similar to but not identical with that produced by insulin. In the latter case, according to the opinions expressed in this paper, a combination of factors is operative. Of these one is the lowering of the blood glucose content, and another is dependent on the formation of glucosone in amounts greater than that normally present. Only the former will be remedied by glucose administration.

Further, in accordance with the view suggested above, glucosone normally will be present in the body at any moment in comparatively small amount, for, as soon as it is produced, it will suffer change partly by transformation into glycogen, partly by being completely oxidised, and partly in connection with the metabolism of fats, as will be referred to later. When extra glucosone is introduced, either by the injection of the compound itself (exogenous glucosone), as in the present experiments, or as the result of insulin administration (endogenous glucosone), the above-mentioned processes will be accelerated. However, there obviously will be a limit to which this acceleration can be carried, and if this limit be exceeded toxic symptoms will inevitably follow as a result of the over-burdening of the tissue cells. This accounts, on the one hand, for the rapidity with which the glucosone effect is manifested, and on the other hand, for the lapse of time before symptoms develop from an insulin injection. It also will be clear that the administration of glucose would not be expected to alleviate the symptoms arising as a result of a subcutaneous injection of glucosone. In the series of experiments in which glucose was injected a little before the glucosone, there was some evidence of a beneficial effect, but this is probably to be explained by the previous glucose injection slowing the absorption of the injected glucosone and thus diminishing its effect.

This same factor, namely, rate of absorption, may also be involved in the experiments in which adrenaline, pitruitrin and also acetoacetic acid were injected before the glucosone, but in these, other and probably more important factors are evidently at work. It is well known that Banting and his co-workers (45) showed that insulin " hypoglycæmia " could be relieved by an injection of adrenaline. This was confirmed by others including Burn, who also discovered the antagonistic action of pituitary extract (46). The experiments now described indicate that it is possible to resuscitate a mouse rendered moribund by an injection of glucosone, by means of either adrenaline or pituitrin ; while by a previous injection of either of these compounds, the glucosone symptoms are inhibited and replaced by another set of symptoms. Those produced in experiments in which adrenaline was used, differed markedly from those occurring after the use of pituitrin. In the former, the high body temperature, the excitability of the animals, and their characteristic wide-awake attitude were striking, while in the latter, drowsiness and inactivity were the features.

As to how adrenaline and pituitrin exert their antagonistic action towards insulin much speculation has already taken place, but it is generally agreed that the mechanism must be different in the two cases. The current opinion that adrenaline promotes liver glycogenolysis would be compatible with the results of the experiments, in which adrenaline was injected either before or after glucosone, the more satisfactory results in the former series being probably accounted for by the adrenaline having time to exert its action quickly enough. Similarly with pituitrin, the increase of inorganic phosphates in the blood noted by Bolliger and Hartman (47), and the fatty infiltration of the liver recently described by Coope (48), may be factors in explaining its effect on the action of glucosone. However, the complete explanation of the action of either adrenaline or pituitrin is not yet known, and further discussion of this question does not fall within the scope of the present paper. Nevertheless, with regard to adrenaline and pituitrin, a close parallelism exists between the effect of insulin and that of glucosone, as ought to be the case if the supposition be correct that glucosone results from the action of insulin.

No doubt a study of the separate fractions of commercial insulin recently isolated by Funk (49) will serve to throw further light on the action of insulin, but in the author's opinion the essential action of the pancreatic hormone is the conversion of $\alpha\beta$-glucose into glucosone, and in the subsequent transformations of the latter compound insulin probably plays no part. This is in agreement with the clinical observations of Thannhauser and Jenke (*loc. cit.*) that diabetics can utilise glucosone. As the result of the successful treatment of a number of diabetics with a preparation of this compound, these workers have advanced a view in some respects similar to that expressed in this paper regarding the part played by glucosone in carbohydrate metabolism. Accordingly, their observations lend valuable support to the author's interpretation of the effects produced on the normal organism when glucosone is administered. Thannhauser and Jenke regard glucosone as an intermediate, which is more readily oxidised than glucose, and conclude that diabetes mellitus results from the inability to form this intermediary product.

They follow Shaffer (35) in regarding the disappearance of the acetone bodies in their cases of diabetes as due to the simultaneous oxidation of the administered glucosone. In connection with this anti-ketogenic action, the experiments with acetoacetic acid, described above, are of interest. It was found that a previous injection of acetoacetic acid into normal mice prevented the development of the "glucosone symptoms," while after the "glucosone effect" had become marked the subsequent administration of acetoacetic acid brought about relief. It would thus appear that glucosone plays an important rôle in the final stages of the metabolism of fat, and also of those amino-acids which yield acetoacetic acid. Whether glucosone forms a compound with acetoacetic acid similar to that which West (50) prepared from glucose, and that the resulting complex is then readily oxidised, must await future investigation. However, as the conception of a complex, which is more easily oxidised than its components, presents certain difficulties despite the electronic interpretation given by West, the alternative view is suggested. One molecule of acetoacetic acid and two molecules of glucosone might interact with the production of two molecules of glucose, one of carbon dioxide and one of pyruvic aldehyde.

$$\mathrm{CH_3.CO.CH_2.COOH + 2CHO.CO.(CHOH)_3.CH_2OH + H_2O} \qquad (a)$$
$$\longrightarrow \mathrm{CH_3.CO.CO.COOH + 2CHO.(CHOH)_4.CH_2OH}$$
$$\mathrm{CH_3.CO.CO.COOH} \longrightarrow \mathrm{CO_2 + CH_3.CO.CHO} \qquad (b)$$

The last-mentioned compound would then by the glyoxalase of the liver become converted into lactic acid and hence into glycogen. This scheme suggests a mechanism for the conversion of fat into carbohydrate, a transformation which, as pointed out by Clutterbuck and Raper (51), is necessary if our present conception of the working of the muscle mechanism is correct, and which, as recently postulated by Wertheimer (52), must ensue as the result of the action of insulin.

It is to be noted that Thannhauser and Jenke, because of the difficulty of obtaining *pure* glucosone in sufficient quantity, used for their work a commercial preparation which contained other substances. As they found that on continued use these impurities produced bad effects such as diarrhœa and gastric disturbances in their patients, they abandoned the employment of glucosone. Feeding mice with the *pure* compound, which has little if any taste, has been attempted but with only partial success, as the animals, even though hungry, refuse to take the substance in sufficient quantity *at a time* to render possible the development of any definite symptoms. As no evident harm resulted to the animals used in these experiments or to animals which on successive days received subcutaneously almost lethal doses of glucosone, the oral administration of the pure compound is not likely to be attended with any deleterious effects. Moreover, Thannhauser and Jenke have already established the fact that glucosone is effective by mouth.

The study of the effect of glucosone on normal mice has yielded highly suggestive results and the work is now being extended to larger animals. This will enable the performance of experiments to establish definitely the suggestions put forward in this preliminary communication, and thus, on systematic lines, to prepare the way for the possible clinical application of the compound.

SUMMARY.

Methods are outlined for the production of glucosone in a purified form.

This material has been found to produce, on subcutaneous injection into mice, a condition similar to that of a so-called "insulin hypoglycæmia."

The physiological activity is specific for glucosone, as no effect is manifested by either lactosone or maltosone.

The striking resemblance of the symptoms produced by glucosone to those caused by insulin, coupled with the fact that their development is modified or inhibited by either adrenaline or pituitrin, indicates a close connection between glucosone and insulin.

The "glucosone effect" is not, however, remedied by the administration of glucose. The reasons for this are discussed.

The possible significance of glucosone in fat metabolism is indicated by the observation that an injection of acetoacetic acid antagonises the effect of a glucosone injection.

It is suggested that glucosone is an important intermediate in carbohydrate metabolism, and that its formation results from the action of insulin on the blood sugar.

In conclusion, the author desires to express his indebtedness to Prof. P. T. Herring for his constant advice regarding the physiological problems connected with the work, and to Principal Sir James Irvine, C.B.E., F.R.S., for his criticism on the chemical aspects. Grateful acknowledgment is also due to Mr. F. M. Weston for much assistance rendered throughout the entire course of this investigation.

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Interaction between Ipsilateral Spinal Reflexes acting on the Flexor Muscles of the Hind-Limb.

By Sybil Cooper, D. E. Denny-Brown (Beit Memorial Fellow), and Sir Charles Sherrington, O.M., F.R.S.

(Physiological Laboratory, Oxford.)

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(Plate 14.)

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Introduction.

The structural scheme of the nervous system involves overlap in the terminal distribution of the afferent arcs converging on the final motor units. A physiological consequence attaching to this extensive overlap, so far as concerns one of its two main categories, forms the subject of the present enquiry.

The convergent junctions which, subserving the 'principle of convergence' (25), result from the overlap, present for functional examination a twofold set of cases. Composing one main class are those cases in which the afferent arcs converging upon the common unit or path tend to act on it to like effect, *i.e.*, in which the reflexes are 'allied' (27). The other class is that where the convergent arcs meeting at the common unit are such as act there to opposed effect, *i.e.*, are 'antagonistic' (27). This latter, namely the class of antagonistic reflexes, in which excitation reacts against inhibition and *vice versa*, has since its formulation been a frequent subject of experimental enquiry and possesses a not inconsiderable literature. On the contrary the case of interaction of 'allied' reflexes has attracted little experimental enquiry and received relatively little attention. In 1909 observations by Camis (8) were devoted to it and revealed features of interest inviting further study. These observations of Camis (8) were dealt with in our recent paper (9), which, while confirming and somewhat extending them, indicated that the problem of the interaction of allied reflexes must be met in the body by some co-ordinative solution playing a great rôle in the reflex taxis of the limb. The present observations had in view to observe the modus operandi obtaining between a typical pair of allied reflexes embouching upon a final path, for which both are excitatory.

METHOD.

As in our previous experiments (9) the preparation (cat) was "spinal" and the test-muscles were three flexors, namely of hip (*tensor fasciæ femoris*), knee (*semitendinosus*) and ankle (*tibialis anticus*). The afferent nerves taken have been those of the limb which provided the test-muscle, and their stimulation has been faradic from two coreless inductoria of similar construction, each fed by somewhat less than 0·2 amp. The isolation and fixation of each test-muscle has been as previously described. The myograph has been that described in a previous paper (11); it records on the isometric principle, with optical registration and magnification of tendon movement up to 550 times.

The plan has been to institute comparison between the contraction evoked by double, *i.e.*, concurrent, stimulation of the two afferent nerves and the contractions evoked singly by the two nerves reacting apart. The compared nerves were armed each with an electrode-pair served by a separately operated coil. Release of keys, worked automatically by the falling piston of the camera, adjusted the time-relations of the stimulations of the nerves. The actual movements of the unshorting and shorting keys were photographed on the myograph record. Time was registered in 0·02 sec. by the Rayleigh magnetic wheel as adapted by Bull. The myograph records are isometric, and the contraction ordinates are rectilinear and vertical. For the purpose in view it was essential that the registration should be free from over-shoot and lag. The myograph employed gave, as reported on previous occasions (11, 9), good evidence of full avoidance of those defects in the most extreme demands here made upon it.

RESULTS.

The three above-mentioned flexor muscles were chosen for observation partly because each of them is excitable reflexly from practically each and every individual afferent nerve of the limb (28). With these muscles and these nerves a general upshot from the observations has been that reflex interaction of one kind or another has been evident in every instance and between every nerve-pair tried whichsoever the pair were, and whichsoever was the test-muscle. In no case has the reflex evoked from either member of the pair been found indifferent to, or for, that of the other member of the pair. Concurrence of the reflexes has always entailed interaction between them.

The form of interaction varies, however, for different nerve pairs, though in all cases assignable to one or other of two main types. These two types of interaction, regarded from the aspect of their net results, are conveniently distinguishable as follows. The two test-reflexes being made concurrent by overlapping sequence (1) in one category of result the concurrent contraction, for one of the two possible directions of the sequence, is less than the contraction on which it supervenes; (2) in the other category, irrespective of the direction of sequence of the two reflexes the concurrent contraction is not less than the contraction on which it supervenes.

Section I.—Inhibition. The two reflexes running their course in overlapping sequence the result given by their concurrence is that, in one or other direction of the sequence, the contraction diminishes with supervention of the concurrence.

A good illustration is the interaction of int. saphen. and poplit. nerves on the hip-flexor, *tens. fasc. femoris*. The popliteal, stimulated during the full reflex contraction evoked by strong stimulation of int. saph. causes relaxation (fig. 1)* so as to annul some five-sixths of the contraction. This reflex inhibition exerted by popliteal attaches mainly to its branches below those to the sural triceps. It acts against the proprioceptives of the front-thigh muscles besides against intern. saph. although less markedly. We have met instances (3 preparations) in which it was not discoverable; but more commonly it has been present, although not in like degree in each case. We have found traces of a similar inhibitory action attach to peroneal also (fig. 17). As to popliteal, not only does it exert the above inhibition on this hip-flexor, but it also, though less powerfully, and much less regularly, causes partial inhibition of the contraction of the knee-flexor, *semitendinosus*, as reflexly evoked by peroneal: this inhibition may annul a third of the reflex contraction. With the ankle-flexor, *tibialis anticus*, we have, however, not found these inhibitory effects either from popliteal or from any other of the ipsilateral limb afferents.

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* In all figures the ordinates are rectilinear, giving tensions; scale to right. Time is above. "Magnif." means multiplication in the original of the tendon movement by the myograph; the size of the original is given by the reproduction of the 1 cm. mark on it. All the figures are tracings from the photomyograms, for the most part much reduced.

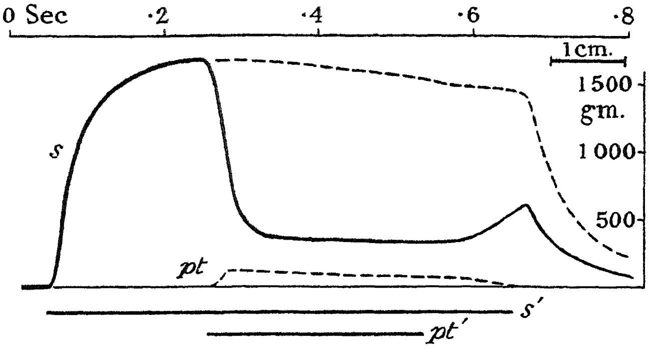

FIG. 1.—Contraction from stim. (13 cm. coil) of int. saphen. n. during s', followed by stim. (13 cm. coil) of popliteal n. during pt'. The broken line above shows course of control reflex from s' alone; the broken line below, course of reflex from pt' alone: *tens. fasc. femoris.* Magnif. 550.

Clearly this restricted ipsilateral flexor inhibition, of sub-genual source, forms a departure from the broad rule (27, 6) that ipsilateral hind-limb reflexes are excitatory and not inhibitory to the flexor muscles. The afferents which exert it conceal it, so to say, under an accompanying excitatory effect; in virtue of this latter they conform to the general rule, and, when tested on the specified flexor at rest or in weak reflex contraction, they so conform without hint of exerting any but excitatory influence, except in some cases by post-stimulation rebound. Thus popliteal unfolds its inhibitory influence on int. saphen. contraction of *tens. fasc. fem.* and on peroneal contraction of *semitendinosus* only when these reflex contractions are at or near maximal; otherwise it excites further contraction in them. In other words its inhibitory field includes among the motor units of *tens. fasc. fem.* excitable by intern. saph., and among the motor units of *semitendinosus* excitable by peroneal, those of high threshold but not those of low threshold. Popliteal, as said above, exerts on *tensor fasc. fem.* more inhibition than on *semitendinosus*, conversely as an excitatory afferent it evokes a larger proportion [64 to 87 per cent. (9)] of contraction from *semitendinosus* than [28 per cent. (9)] from *tens. fasc. fem.* For an excitatory and an inhibitory reflex influence on the same muscle to attach admixed to one and the same afferent is not uncommon : it has frequently been observed in regard to extensor muscles (29, 19) ; the present instance extends it to certain of the flexors. As to the residue of contraction which remains uninhibitable, even under maximal stimulation of the popliteal, there is some evidence that this residue is partly made up of the same motor units which are excited by popliteal itself and yield the contraction which accompanies the inhibition and is obviously irrepressible by it.

One feature of the inhibition is its remarkable post-stimulation persistence (fig. 2A). The inhibitory effect long outlives the excitatory. Even when its stimulus has been weak and brief (*e.g.*, 0·1 sec.) and the consequent inhibitory relaxation moderate, the excitatory nerve (int. saph.) may yet be unable for a full second or more to restore fully its lost contraction. If the inhibitory

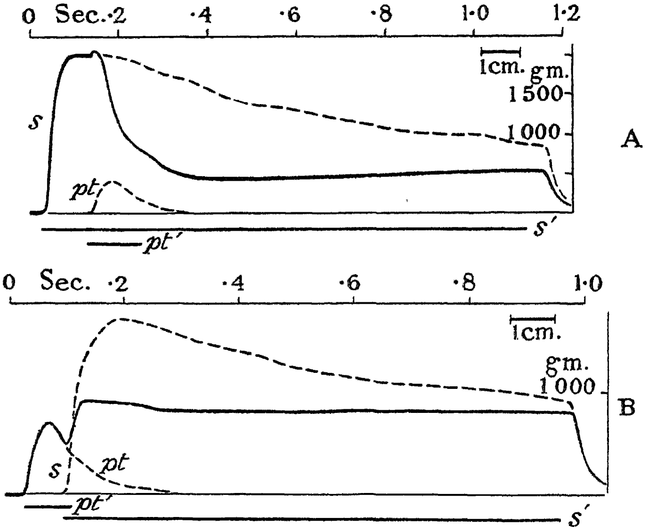

FIG. 2.—A. Contraction from stim. (13 cm. coil) of int. saph. during *s′*, followed by stim. (13 cm. coil) of poplit. during *pt′* causing mixed but predominantly inhibitory effect. Broken line above shows control reflex from *s′* alone ; broken line below, the reflex from *pt′* alone.

B. Similar to A, but *pt′* precedes *s′*. *Tens. f. femoris.* Magnif. 550.

(popliteal) reflex be precurrent (fig. 2B) the after-coming excitatory (int. saph.), although provoked subsequent to withdrawal of the inhibitory stimulus, is dwarfed in size. The dwarfed reflex maintains a strikingly level plateau course. Its horizontality, contrasting against climax and declining course of the control undwarfed reflex, indicates that the inhibition has selectively weeded out the elements of briefer response, leaving a relatively homogeneous residue of units of long-lasting reaction. This recalls the selective inhibitory suppression of motor units of briefer reaction and after-discharge in the crossed extension reflex (20).

As to the functional meaning of this inhibitory spinal reflex, exceptional in its feature of acting on ipsilateral flexors, examination showed that it can be accompanied by contraction of the ipsilateral knee-extensor, not of powerful degree, but prompt and effectual. The inhibition of the hip-flexor thus appears as a reciprocal reflex-accompaniment of reflex extension of the knee. It is possible that this reflex is related to the extensor thrust, the ipsilateral hind-limb extension reflex seen so characteristically in the spinal dog (27). Or it may well be the inhibitory counterpart of the "magnet reaction" or indeed the whole "positive supporting reaction" of Magnus (22, 23) seen in the hind-limb of the decerebellated animal.

However that may be, this ipsilateral spinal reflex partially inhibiting contraction of the hip-flexor, *tensor fasciæ femoris*, and in less degree and with less regularity of the knee-flexor, *semitendinosus*, constitutes but a minor portion of the ipsilateral reflex reaction elicitable from the limb. The major portion is the well-known reflex which is excitatory of the limb flexors and inhibitory of the limb extensors (27). This predominating reflex reaction is customarily spoken of as a single reflex, the "flexion reflex"; under one name is thus embraced a numerous collection (11) of similar but not identical reflexes, all of them "allied" in as much as excitatory of the same flexor muscles though not each in like degree. It is to this large group of allied reflexes that our observations have turned for examination of the mode of interaction obtaining between spinal reflexes activating one and the same final common path.

Section II.—Concurrence of the two reflexes yields a contraction not less, and usually greater, than that of the larger of the two component reflexes taken separately.

§ 1. *Occlusion.*—The concurrent contraction is less than the sum of the component contractions observed separately.

(1) The reflexes tested for interaction are from tributary branches of the same nerve.

The posterior tibial nerve's two plantar branches, stimulated together across a single pair of electrodes with current strength found maximal for each, evokes in *tibialis anticus* a contraction which falls much short of the sum of the two contractions obtained by applying the electrodes and stimulation in turn to each of the two nerve-branches singly (fig. 3). The deficit is not due to over-valuation of the singly evoked component contractions by fling of the recording system (*cf.* p. 264 above); nor to escape of stimulating current from one branch to the other, fuller isolation of the branches making no difference to it (*cf.* also next sub-section). Further the deficit still occurs when the two branches are maximally stimulated singly from separate circuits, and are then, by synchronising their two stimulations, both excited concurrently without change of circuit-resistances or electrodes. Observed in either way the reflexes when concurrent suffer extinction of part of their reflex effect.

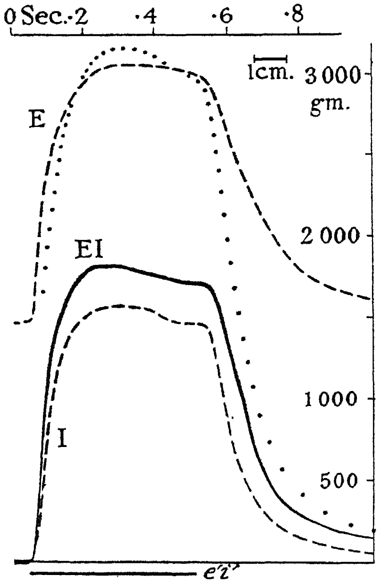

FIG. 3.—Contraction from stim. (11 cm. coil) of both plantar nerves during *ei′*. Upper broken line* contract. from stim. (13 cm. coil) of extern. plant. alone ; lower broken line, from stim. (13 cm. coil) of intern. plant. alone. Dotted line indicates course of contraction if E and I had fully added themselves. *Tibial. anticus.* Magnif. 205.

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* The one component reflex is supposed based at the level of the climax of the other, to indicate the potential summation. In figures which follow, the post-current reflex is shown based at the level reached by the pre-current when the post-current set in.

As to which of the two components suffers the curtailment an indication is obtained by evoking one, *e.g.*, plantar I (fig. 1B, Plate 14), for a short intercurrent period during the plateau course of the reflex from the other, plantar E. The intercurrent reflex then adds, for the period of concurrent stimulation and for somewhat longer owing to after-discharge, an increment (fig. 1B, Plate 14) to the contraction of the other. The increment given thus by I is not so large as the contraction evoked by I when not concurrent with E (fig. 1B, Plate 14). And when the sequence is reversed, E following on I (fig. 1A, Plate 14), it is the reflex contraction of E that exhibits the deficit. The deficit is far too large to be accounted for by the initial-tension disability of a partial contraction following, in the same muscle, on a partial contraction, as noted by Fulton (17). In the case of the two plantars, by weakening even slightly the stimulation for the intercurrent reflex in relation to the precurrent it is possible so to adjust them that in one order (fig. 1A, Plate 14) of sequence the intercurrent reflex E fails to add any increment at all to the precurrent contraction, and seemingly defaults altogether (I + E, fig. 1A, Plate 14). Reversal of the sequence then shows that though total suppression of the intercurrent reflex occurs in one direction of the sequence it does not occur in the other, E + I. Therefore if the contraction of the after-coming reflex be regarded as that which suffers curtailment from the concurrence, the order followed by the sequence, *i.e.*, whether E follows I or *vice versa,* is not a matter of indifference to the quantitative result.

A similar kind of interaction obtains with other pairs of tributary branches common to a parent nerve. Thus, with the dorsal digital branches from musculo-cutaneous division of peroneal as tested on *semitendinosus* muscle (fig. 4A, and fig. 2, Plate 14), with intern. saphenous and the sartorius branch of anterior crural trunk tested on *tens. fasc. femoris* (fig. 4B), and similarly with the superficial and deep branches of obturator.

(2) The reflexes tested for spinal interaction are from afferent nerves not branches of the same parent nerve.

With nerves anatomically wider apart than those dealt with in sub-section (1), risk of current-spread from one to the other can be discounted altogether. The following observations therefore, while extending the results of sub-section (1), add confirmation to them.

> Thus, *tib. ant.* muscle; reflex contraction from ext. plantar n. 1130 gm.; from n. of hamstring muscles, 1050; the latter timed for concurrence with the plateau course of the former added 420 gm., *i.e.*, a deficit of 60 per cent. contraction. Similarly, the contraction evoked by the

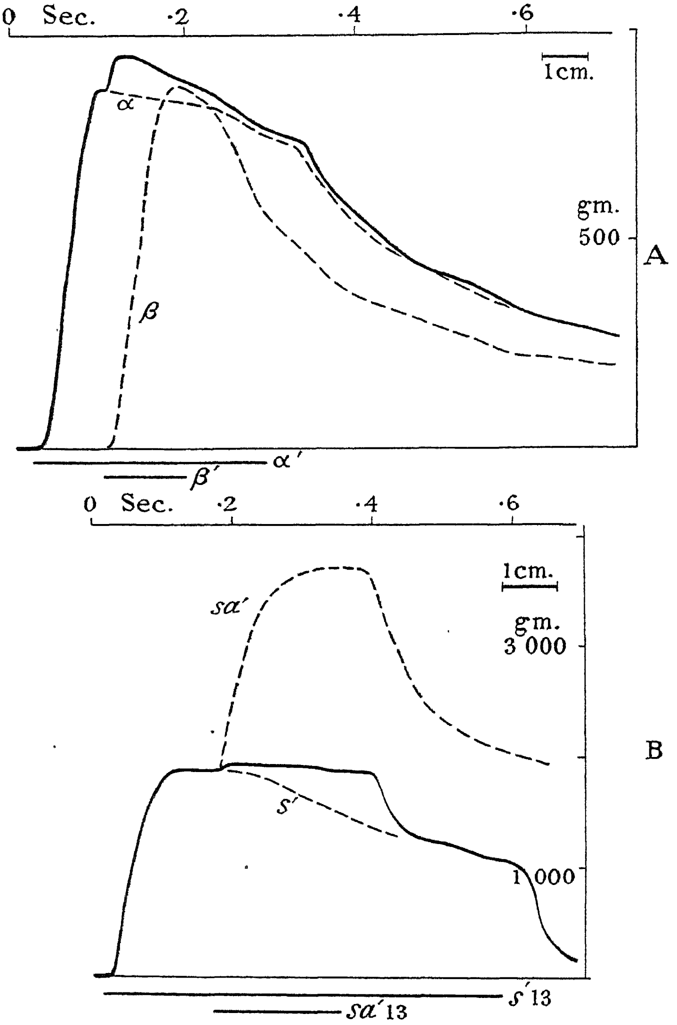

FIG. 4.—A. Contraction from stim. (13 cm. coil) on dorsum of foot of nerve branch to 2nd digital cleft during α′ followed for a short intercurrent period β′ by stim. (13 cm. coil) of a similar nerve-branch to 3rd digital cleft. Broken line α shows control reflex for α alone; broken line β that for β alone. β's reflex when intercurrent on α's exhibits a contraction-deficit of more than 90 per cent. *Semitend.* Magnif. 550.

B. Contraction from stim. (13 cm. coil, 95 per second) of int. saphen. n. during *s′* following during intercurrent period *sa′* by stim. (13 cm. coil, 88 per second) of n. of *sartorius*. Upper broken line, control reflex of *sa′* alone; lower broken line shows course of control reflex of *s′* reflex alone. No evidence of interference beats in the concurrent contraction. Contraction deficit of over 90 per cent. *Tens. f. femoris.* Magnif. 550.

hamstring nerve showed 78 per cent. deficit when concurrent with that of intern. plantar (fig. 11A).

Again, with *semitendinosus* muscle, the contraction evoked by int. saph. showed a deficit of 75 per cent. when concurrent with that from peroneal (fig. 5A).

Again, with *tens. fasc. fem.* muscle the contraction evoked by peroneal showed a deficit of about 70 per cent. when concurrent with that evoked from int. saphen. nerve (fig. 5B).

Interaction of this kind with resultant deficit of contraction obtains between limb-afferents as various as the following: peroneal and popliteal, external cutaneous nerve of groin and hamstring nerve, popliteal and quadriceps nerve, internal saphenous nerve and small sciatic, obturator nerve and ileal nerve, etc. It holds for them as tested in regard to each of the three test muscles, except that in *tens. fasc. fem.* it is subject to the condition, frequently though not regularly met with, described in Section I, and to which reference will be made again (Section III).

Clearly this kind of interaction is not confined to tributary branches of one and the same parent nerve. It obtains between nerves belonging to wholly, and even widely, separate parts of the limb plexus and derived from wholly different spinal segments.

(3) The reflexes tested for interaction are from afferent nerves of assorted functional character.

In the above examples one or other individual of the nerve-pair tested was of mixed afferent composition. The following are instances with pure skin or pure muscular afferents chosen for the pairs, or pure skin nerve paired with pure proprioceptive.

Skin nerves: (*a*) int. saph. followed by extern. cutaneous of groin gave 80 per cent. deficit of latter on *tens. fasc. fem.*, or in another example (fig. 12C) 66 per cent.

(*b*) A dorsal digital nerve followed by a similar nerve from next digital cleft gave 88 per cent. deficit of latter on *semitendinosus* (Plate 14, fig. 2); these digitals, though not to be regarded as purely cutaneous (30), are certainly similar in function one to another.

Muscle nerves: (*a*) n. of quadriceps followed by n. of sartorius gave 90 per cent. deficit of latter on *tensor fasc. fem.* (*b*) One division of n. of quadriceps followed by the other division gave 55 per cent. deficit of latter on tens. fasc. fem. A similar experiment (another preparation)

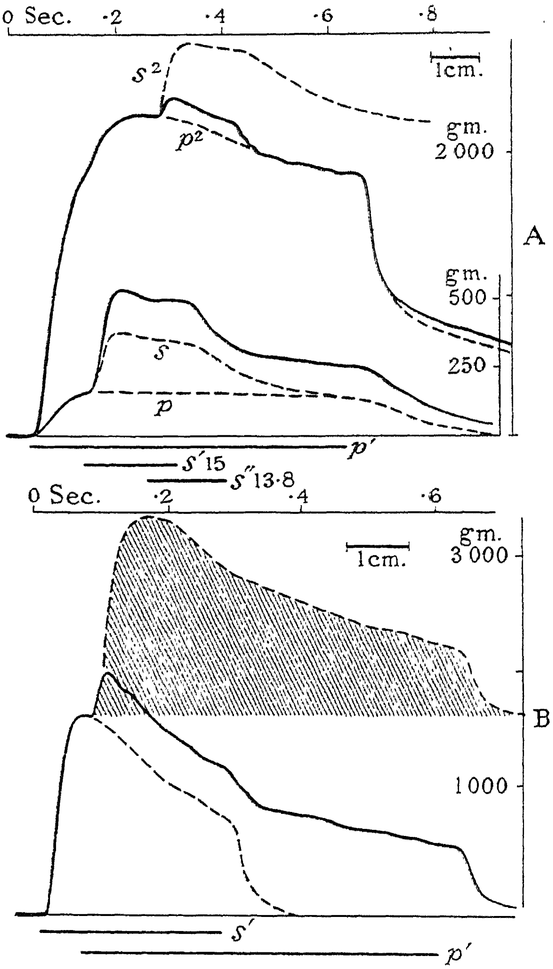

FIG. 5.—A. Contractions of *semitend.* elicited by stim. of peroneal n. during p' followed for a short intercurrent period by int. saphen. n. In the lower obs. peron. stim. 19 cm. coil and int. saphen. stim. 15 cm. coil during s'; the broken lines show the control reflexes, indicating that peron. at this strength has facilitated int. saphen. at given strength. In the upper observation peron. stim. 14 cm. coil, and int. saphen. stim. 13·8 cm. coil during s''; broken line p^2 shows control reflex of peron. alone; broken line s^2 shows contraction from s'' alone; at this strength peroneal occludes more than 75 per cent. of int. saphen. at its strength. Magnif. 200 for lower obs.; 370 for upper obs.

B. Contraction of *tens. f. fem.* elicited by stim. (13 cm. coil) of int. saphen. n. during s', followed by stim. (13 cm. coil) of peron. n. during p'. Upper broken line and hatched area shows control reflex of p' alone; lower broken line, control of s' alone. The post-current reflex is the larger; and emerges from the heavy occlusion little modified to judge from its subsequent course compared with control. Magnif. 550.

with *semitendinosus* as muscle gave 60 per cent. deficit (fig. 6). Observations with the ext. and int. nerves of sural triceps on *semitendinosus* evidenced less deficit.

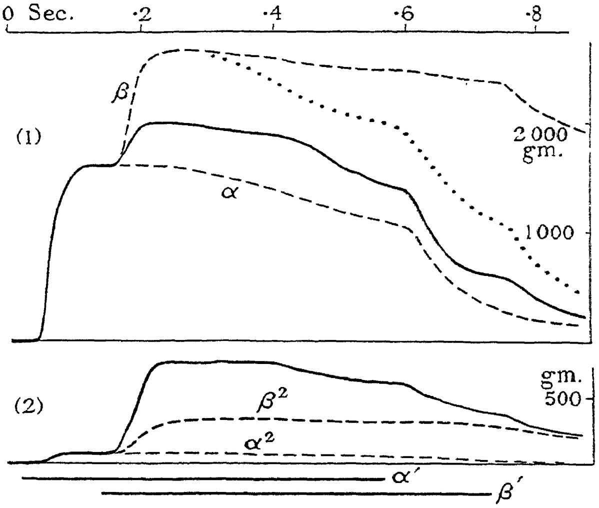

Fig. 6.—Contraction from stim. (95 per second) of deep portion α of n. of *quadriceps* muscle during α′, followed by superfic. portion β (at 88 per second) during β′. In upper observation (1) both α′ and β′ are at 13 cm. coil; in lower observation (2) α′ is at 18 cm. coil, β′ at 16·5 cm. Broken lines show the course of the controls of the component reflexes. The dotted line indicates the course of contraction had α and β simply summed. In upper observation β shows contraction deficit of more than 50 per cent.; in lower, β appears reinforced 50 per cent. *Semitend.* Magnif. 550.

Skin afferents with muscle afferents: int. saphen. followed by sartorius n. gave 94 per cent. deficit of the latter on *tens. fasc. fem.* (fig. 4B); followed by n. of hamstring muscles it gave 76 per cent. deficit of the latter (fig. 7A); ext. cutan. of groin followed by n. of hamstring muscles gave 80 per cent. deficit of the latter. Combined n. (proprioceptive) of quadriceps and sartorius followed by internal saph. (cutaneous) caused total disappearance of the latter's contraction (fig. 7B); and n. of sartorius (proprioceptive) followed by ileal (cutan.) of groin nearly complete deficit of the latter (fig. 12B).

One and the same cutaneous nerve, int. saphenous, tested in the same experiment for interaction with, in succession, a cutaneous nerve (ext. cutaneous of groin), a proprioceptive nerve of a neighbouring extensor muscle (quadriceps exterior) a proprioceptive nerve of a neighbouring flexor muscle (sartorius) and a proprioceptive from segmentally distant muscles (hamstring group) reacted not markedly differently with each in turn, occasioning in each considerably more than 50 per cent. contraction deficit. The mode of reflex inter-

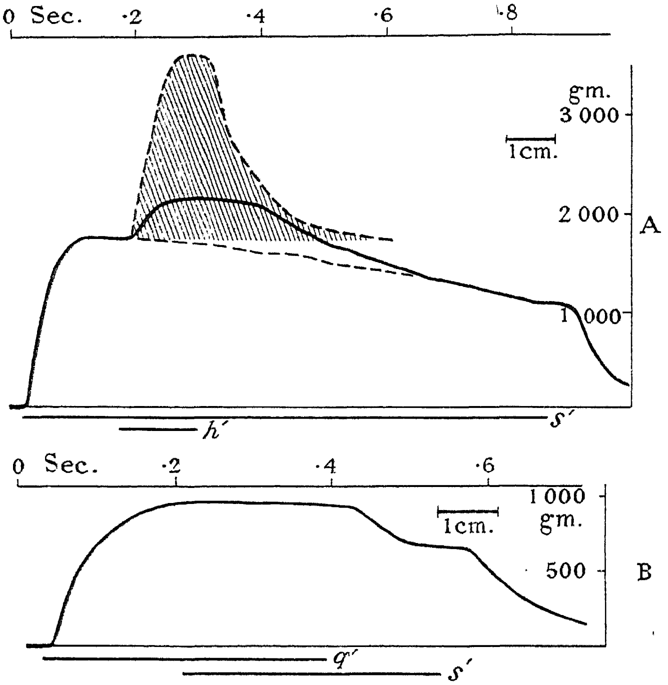

Fig. 7.—A. Contraction from stim. (13 cm. coil) of int. saph. during *s′* followed for a short intercurrent period by n. of hamstring muscles (13 cm. coil) during *h′*. Upper broken line and hatched area shows control reflex from *h′* alone; lower broken line, control from *s′* alone; *h′* reflex shows contraction deficit of 74 per cent. Magnif. 550.

B. Contraction from stim. (12 cm. coil) of combined proprioceptive n. of quadriceps and sartorius during *q′*, followed by int. saphen. (12 cm. coil) during *s′*. Int. saph. causes no contraction-increment at all, yet, after withdrawal of *q′* maintains 70 per cent. of the contraction. Magnif. 210. Both A and B are *semitend.*

action in question evidently applies generally between the afferents thus tested. Its upshot, the default of the concurrent contraction from the sum of the potential component contractions, is of regular occurrence.

For brevity of distinction the defaulting contraction, *i.e.*, the portion missing from the concurrent contraction, will in what follows be termed the *occluded* contraction; and this without prejudice to later discussion as to the nature, whether inhibitory or other, of the process which produces it.

(4) *Amount of occlusion.*—Numerical values for the above observed occlusions have, in citing the observations, been for the most part mentioned; such individual numbers, however, possess but limited significance even for the

occlusions to which they refer. Thus, as can be seen in the myograms figured, the numerical value stated for the occlusion is applicable only to a part of it because the occlusion commonly presents a shift in value as its course proceeds (figs. 4B, 7A, 10, 14A). Also, the occlusion varies in amount not only as between different afferents but also as between a given pair of afferents under different conditions. A factor influencing the amount is strength of stimulus. With stronger stimuli of the paired nerves the occlusion tends to be, relatively as well as absolutely, greater than with stimuli less strong, *e.g.*, *tibial. ant.* muscle; int. plant. n. at 13 cm. (about maximal) followed by ext. plant. n. at 13 cm. occluded 94 per cent. of latter; whereas with int. plant. at 19·5 cm. and ext. plant. at 17 cm. less than 20 per cent. was occluded (fig. 8).

With the paired reflexes in overlapping sequence, stronger stimulation of the

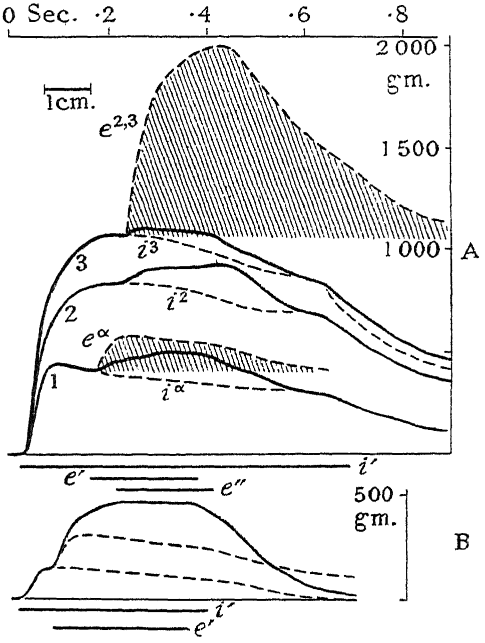

FIG. 8.—A. Stim. of int. plant. n. during i', for 1 at 19·5 cm. coil, for 2 at 15 cm., for 3 at 13 cm., followed by stim. of ext. plant. n. at 17 cm. coil during e' in concurrence with int. plant. 1, and giving $e\alpha$ when alone; at 13 cm. coil during e'' in concurrence with int. plant. 2 and 3 respectively, and giving $e^{2,3}$ (shown with hatched area) when alone. Broken lines i^{α}, i^2, i^3 show course of control reflexes from int. plant. n. The occlusion is relatively as well as absolutely larger with the stronger stim. of int. plantar n.

B. From same expt. as A. Stim. of int. plant. n. during i' at 21 cm. coil, followed by stim. of ext. plant. during e' at 16·5 cm. coil. Lower broken line, control observation of i' alone; upper broken line, control observation of e' alone; pre-current i' seems to have facilitated for e'. *Tibialis anticus.* Magnif. 550.

"leading" reflex, the post-current reflex remaining unaltered, gives greater occlusion.

> Thus, with *semitendinosus* muscle, poplit. n. at 15 cm. occluded 50 per cent. of post-current int. saphen. n. at 14 cm.; at 12 cm. it occluded 66 per cent. of int. saph. With *tibial. ant.* muscle, int. plant. n. as pre-current at 15 cm. occluded less than 80 per cent. of ext. plant. at 13 cm.; whereas at 13 cm. it occluded nearly 95 per cent. (fig. 8). With *tens. fasc. fem.* muscle int. saph. at 15 cm. occluded some 70 per cent. of post-current poplit. at 12 cm.; but int. saph. at 13 cm. occluded the whole of it (fig. 15).

Conversely, the leading stimulus remaining unaltered, its reflex occludes more of the post-current reflex when the latter's stimulus is weakened.

> Thus, *semitendinosus* muscle; poplit. n. as pre-current at 11 cm. occluded 31 per cent. of peroneal at 13·8 cm., but 50 per cent. with peron. at 19 cm. Again, *tibial. ant.* muscle; poplit. n. at 13·8 cm. as post-current reflex was occluded 50 per cent. by peron. n. at 18 cm.; 70 per cent. at 17 cm.; and 95 per cent. at 14 cm.

The strength of the stimulus influences the amount of occlusion, and the key to its importance in that respect is its influence on the size of the reflex contraction. Thus, toward end of a lengthy experiment when the strength of stimulation which earlier evoked ample reflexes provokes less ample ones, the amount of occlusion similarly falls off.

Again, of occlusions exerted between the plantar nerves, with *semitendinosus* as test muscle, the largest we have observed, namely, 98 per cent., was observed after strychnine (in sub-convulsive dose), a drug under which the fractional group of the muscle's units proper to each individual afferent is much enlarged (9).

With concurrence of the reflexes arranged by overlapping sequence, the amount of occlusion is conveniently expressed as a fraction of the post-current reflex's independent contraction, this latter being found separately by control observation. Thus expressed the numerical value for the occlusion obtaining between a given pair of afferents will vary according as the larger reflex is pre-current or the smaller one.

This, of course, does not mean that the direction of the sequence of the reflexes determines whether the occlusion shall be more or less. We have no evidence that the order of their sequence affects quantitatively the occlusion between two reflexes. If such influence exists it has been too slight or too inconstant to be clearly apparent.

The amount of occlusion occurring between a given pair of afferents is not the same for the several test-muscles. Thus popliteal produces less occlusion as tested on *tens. f. fem.* than on the other two muscles. Peroneal is a particularly powerful occluder in *semitendinosus.* On the other hand, the occlusion between ext. and int. plantar nerves has been with *tibialis anticus,* fairly similar in percentage amount of its reflex to that observed with *semitendinosus,* both by the simultaneous method and by that of overlapping sequence. Comparison of the occluding power of different afferents on the several muscles shows broadly a proportionality between it and reflex-producing power as judged by amplitude of resulting reflex, *i.e.*, by the size of the fraction of muscle which it can reflexly activate (11, 9).

In some instances, with overlapping sequence of the reflexes, their concurrence occasions nothing whatever in the way of increment of contraction; the post-current reflex is occluded totally. "Total occlusion" between reflexes which are approximately equal in size, is, to say the least, infrequent. Thus, with *tens. f. fem.* muscle, when the reflexes obtainable by full stimulation of peron. n. and of int. saphen. n. respectively are, as is often the case, nearly balanced in size, total occlusion of the one by the other, whichever way their sequence be taken, we have not obtained, although occlusion has reached 90 per cent. But in experiments where peroneal reflex was not inconsiderably the larger, peron. has totally occluded int. saph. under full stimulation of both; and conversely in experiments where int. saphen. n. yielded the considerably ampler reflex that of peroneal has been totally occluded by it. Total occlusion can, with overlapping sequence of the two reflexes, document itself in two ways: by (1) with the larger reflex precedent, the smaller giving no increase to the contraction on which as post-current it supervenes (figs. 9, 7B, 13, 15A; fig. 1A, Plate 14); and by (2) with the smaller reflex precedent, its withdrawal occasioning no decrement of the contraction which, under the post-current reflex alone, continues unchanged (figs. 10A, 11B).

The two plantar nerves as tested on *tibialis anticus* and *semitendinosus,* and the dorsal digital nerves tested on *semitendinosus,* have proved those which, each individually evoking approximately equal reflex contractions, have under full stimulation of both come nearest to yielding when concurrent total occlusion of the contraction of one by the other. Thus, between the plantars there are occlusions of even 94 per cent.* and 95 per cent. (fig. 3); and between the dorsal

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* These numerical estimates, though precise for the myograms, do not claim exactness for the muscular units; for instance, they assume these latter to be all of one standard value in terms of contraction-tension. And *cf. supra,* p. 269.

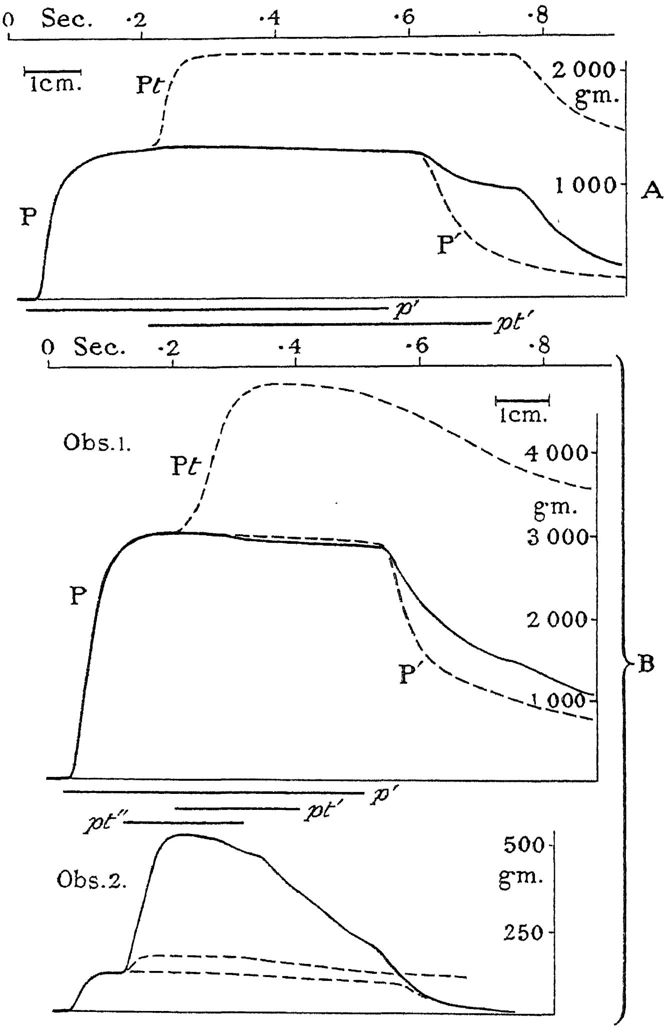

Fig. 9.—A. Contraction (P) elicited by stim. (11 cm. coil, 95 per second) of peroneal n. (except branch to tibialis anticus) during *p′*, followed by stim. (13 cm. coil, 88 per second) of popliteal n. during *pt′*. Broken line P*t* above shows control reflex from *pt′* alone; broken line P′ shows end of control reflex from *p′* alone. Popliteal gives merest trace of increment. The plateau of the concurrent reflex is steady although rates of stimulation interfere with a period which would be easily registered by the myogram. *Tib. antic.* Magnif. 550.

B. Contraction (P) elicited by stim. of peroneal n. during *p′*, in obs. 1 at 16 cm. coil, followed by stim. of popliteal at 15 cm. coil during *pt′*. Upper broken line *pt* shows control given by *pt′* alone; lower broken line P′ shows control reflex given by *p′* alone. Peroneal reflex totally occludes that of popliteal, though latter (*cf.* control) develops, when by itself, some 1750 gms. tension. In obs. 2 peroneal stim. is as in obs. 1, except that coil distance is 25 cm.; it is followed by popliteal stim. *pt″* at 21 cm. coil. Upper broken line control observation of *pt″*'s contraction when alone. Lower broken line, control reflex of peroneal alone at 25 cm. coil. Popliteal instead of being submerged by occlusion as in obs. 1, adds an increment to peroneal larger than its own control contraction, *i.e.*, is enhanced instead of occluded by pre-current peroneal. *Semitendinosus.* Magnif. 480 in obs. 1; 200 in obs. 2.

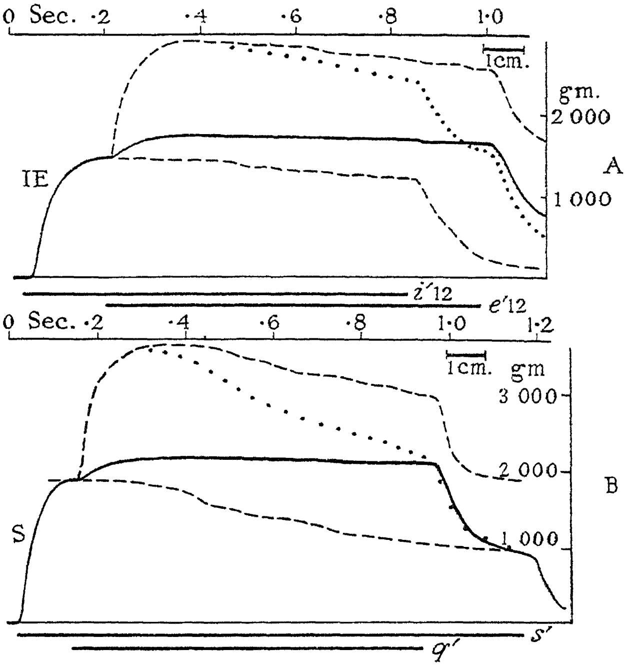

FIG. 10.—A. Contraction (IE) elicited by stim. (12 cm. coil, 94 per second) of int. plantar n. during *i′*, followed by ext. plantar n. (12 cm. coil, 83 per second) during *e′*. Lower broken line shows control reflex from *i′* alone; upper broken line, control from *e′* alone. Dotted line indicates course of contraction had *i′* and *e′* reflexes simply summed. At emergence from concurrent period the plateau level falls very little, post-current reflex maintaining almost the whole contraction. The plateau of the concurrent period shows no undulations corresponding with the interference beats of the rhythm-rates of the concurrent stimuli. *Tib. anticus.* Magnif. 550.

B. Contraction S elicited by stim. (13 cm. coil, 95 per second) of int. saphen. n. during *s′*, followed by n. of quad. extensor (13 cm. coil, 88 per second) during *q′*. Upper broken line, control reflex of *q′* alone; lower broken line, control of *s′* alone. Fall after *q′* indicates that during concurrence the motor units largely changed hands; the concurrent period's plateau shows no undulations at interference rate of the stimuli. *Tens. f. fem.* Magnif. 550.

digitals similarly (fig. 4 shows 90 per cent.). The amount of occlusion between the two plantar nerves seems, especially in *semitendinosus* muscle, greater than that to be expected from the size of their proper fractional reflexes.

On the other hand by pairing nerves of unequal strength of reflex effect 'total' occlusion of the contraction of the less powerful by the more powerful is in many cases readily obtained, even though the less powerful be itself a nerve of large reflex effect. Thus, peroneal is powerful on *tens. f. fem.*, but, as mentioned above, int. saphen. in some experiments can occlude it totally, and commonly occludes popliteal totally (fig. 15A). Further, the large proprioceptive afferent from the front thigh muscles may totally occlude the maximal reflex contraction of even internal saphen. n. itself (fig. 7B). Again with *tibialis anticus* muscle, the int. plantar n.; although evoking a reflex, of 1250 gm. was occluded totally by musculo-cutaneous branch of peroneal (fig. 11B). Since int. plantar can of itself occlude 95 per cent. of ext. plantar's reflex, musculo-cutaneous should occlude totally the maximal reflex contraction of both plantars combined so far as ankle flexor *tibialis anticus* is concerned. Again, popliteal, although a very powerful afferent for *tib. anticus*, can have its contraction totally suppressed in that muscle by occlusion from peroneal (fig. 9A). The afferent peroneal nerve is an extremely powerful occluder, perhaps especially in *semitendinosus* muscle. Fig. 9B shows it totally occluding a popliteal reflex of over 1·7 kil. pull. In another experiment, with semitendinosus, it totally occluded the reflex contraction, value 1550 gm. due from maximal stimulation of the two plantar nerves stimulated as one trunk above the ankle (fig. 13). On the other hand we have seen maximal stimulation of the peroneal and popliteal nerves in combination fail to effect total occlusion

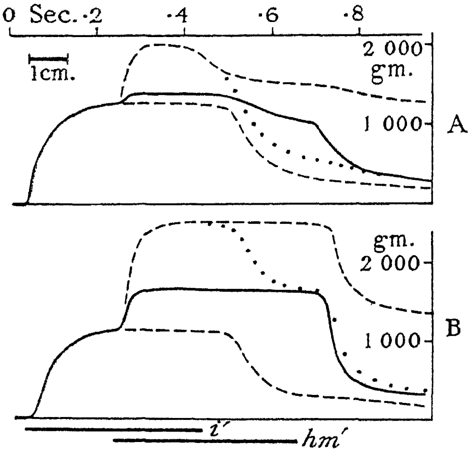

FIG. 11.—A and B. Contraction evoked by stim. (12 cm. coil, 90 per second) of int. plantar n. during *i′* followed in upper myogram by hamstring n. (12 cm. coil, 80 per second) during *hm′*, in lower myogram by musculo-cutaneous br. of peroneal (12 cm. coil, 80 per second) during *hm′*. Broken lines show course of the controls of the pre-current and post-current reflexes respectively. Dotted line indicates the course of the contraction had the post- and pre-current reflexes simply summed. In upper myogram the post-current reflex issues from the concurrence improved in staying power. In lower myogram the post-current reflex occludes the pre-current almost totally. *Tib. antic.* Magnif. 550.

of the reflex contraction from maximal stimulation of int. saphenous. Such variations from experiment to experiment have been, however, only quantitative, and not in fact extreme. They are such as experience of spinal fluctuation (9) in the size of an afferent nerve's proper fractional motor-unit group might expect. They do not obscure the broad upshot that the reflexly available size of that group is a main determinant of the amount of occlusion in interaction with other afferents allied in kind.

(5) *Time relations of reflex occlusion.*—Occlusion begins to take place from the very outset of the period of concurrence. Its latency period is often remarkably short, shorter in some cases than that of the control reflex itself. It commonly reaches its climax of mechanical (isometric) effect much earlier than does the control independent reflex contraction, that is, the contraction-increment attains its plateau-height earlier than does the contraction of the unoccluded reflex as seen in the control observation (fig. 4A). Where the two interacting reflexes are excited simultaneously, maintained (not for too long) together, and have their stimuli withdrawn together the degree of occlusion throughout the plateau-period alters little (fig. 3). It increases smoothly during the ascent, and decreases similarly though more slowly during the post-stimulation after-discharge phase. It is, of course, impossible from such an observation alone to know to which of the two components to tax the deficit, or whether to regard it as shared by both.

Where the concurrence is obtained by supervention of a post-current reflex in course of an already developed pre-current one, the occlusion opens ordinarily with greater intensity than is maintained (figs. 4B, 10B), the contraction-increment behaving as a sort of reciprocal of the occlusion's value. This is often marked under a protracted concurrence when the total reflex contraction declines. Any cause by which the predominant reflex, as it proceeds, becomes weakened tends to lessen the occlusion.

(6) *The occlusion is not due to inhibition.*—The foregoing observations taken together make out a case for existence of a characteristic interaction between allied excitatory reflexes, an interaction proper to them as a class. One of the points evidenced is that when two such reflexes, each evoking contraction of one and the same limb muscle are excited concurrently the resulting contraction shows, as Camis (8) established for popliteal and peroneal nerves on semitendinosus, a deficit from the sum of the potential contraction of the two separate reflexes. As to how it comes about that the interaction produces this deficit, the following considerations arise.

Assuming overlapping sequence of two excitatory reflexes A and B, and turning first to the contraction increment obtained from B's concurrence with A, that increment, if the all-or-none principle be accepted for the motor units and muscle fibres concerned, is referable not to any further enhancement by B of the contraction already in progress in units activated by A, but to B's activation of fresh units additional to those activated by A. This interpretation is upheld by actual observation.

> Thus: A's reflex, excited by a shock series of 76 per second, gives a contraction with faint indications of the stimulus rhythm; B's reflex, excited by a shock series of 43 per second, gives a contraction with well-marked waves corresponding with that slower stimulus rhythm. On B's reflex becoming concurrent with A's, an increment of contraction occurs amounting to somewhat less than 30 per cent. of B's full value. The combined reflex during this increment would be expected, were the increment due to enhanced and accelerated contraction of units already responding to 76 per second rhythm [A's reflex] to show either a confused, irregular rhythm, or no rhythm at all, the conjoint rate of 119 per second being somewhat too quick for the mechanical response of the muscle-fibres to reveal. Neither of these results in fact happens. What happens is that the contraction of the combined period shows a fully-marked and unimpeded 43 per second rhythm which (B's stimulus still being maintained after withdrawal of A's) proceeds without break into B's portion proper of the reflex. Again, with a stimulus rate of 220 per second for A's reflex and of 35 per second for B's, on B's reflex adding increment even as low as 7 per cent. to A's contraction, that increment at once and throughout showed the ripple of the 35 per second rhythm of B's excitation (fig. 12). B could not have effected this as an enhanced reaction of units already reacting to stimuli at 220 per second. B's increment must be due to fresh other units reacting at 35 per second. Again, with A's stimulus at 50 per second and B's at 220 per second, the concurrent contraction on supervention of B showed 50 per second rhythm continuing unswamped even though more than half of the concurrent contraction was due to B's increment at 220 per second. The increment was obtained, therefore, without engaging muscle-fibres already reacting to A at 50 per second.

Turning now from the increment, *i.e.*, the unoccluded portion of the contraction, to the missing, 'occluded' portion, we may consider first the case of a 'partial occlusion.' Comparison with the control reflexes indicates that the amount of the increment often increases as the concurrence proceeds while the amount of the occlusion contemporaneously diminishes, as though the two were complemental and the increase of the one at the expense of the other. The two features may therefore be considered together.

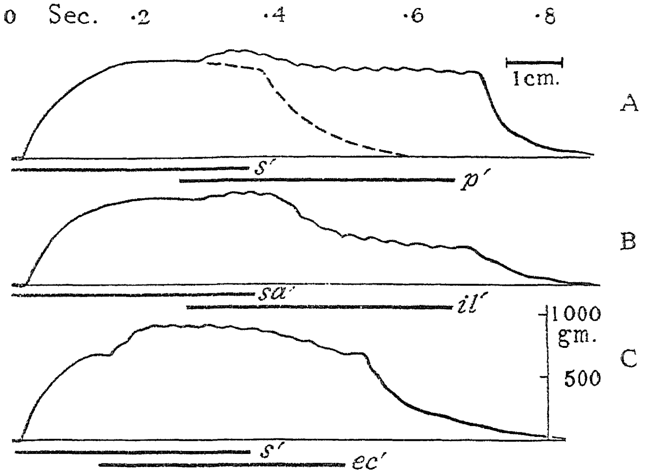

Fig. 12.—Contraction: A, by stim. (12 cm. coil, 220 per second) of int. saphen. n. during s' followed by peron. n. (12 cm. coil, 35 per second) during p'; broken line shows course of control reflex from s' alone. B, by stim. of proprioceptive of *sartorius* (13 cm. coil, 220 per second) during *sa′*, followed by ileal cutaneous n. (13 cm. coil, 35 per second) during *il′*. C, by stim. (16·5 cm. coil, 220 per second) during s' followed by ext. cutan. (15 cm. coil, 35 per second) during *ec′*. *Tens. f. fem.* (post. portion). Magnif. 390. Scale of tension the same for all.

As to the occlusion itself explanations might be alternatively offered as follows:—

(1) A motor unit while activated, tetanically and at sufficient frequence from one source, might be incapable of additional effective activation from another source; and its activation by the first source might conceivably remain in operation without sustaining obvious disturbance from the second. For reflex action the occurrence of a precluding relation of this kind has been already (15, 19) advanced in explanation of cases where motor nerve-fibres have seemed to be pre-occupied by reflex after-discharge. (2) Instead of appeal to so scantily known a process as the above, explanation might be sought on the well-known lines of reflex inhibition, such, for example, as competing antagonistic reflexes commonly exert in instances often studied.

Of these alternative lines of explanation the latter, the inhibitory, may be considered first. Resort to inhibition has to acknowledge in the present case the preliminary disability that reflex inhibition, so far as instanced elsewhere, attaches to reflexes which although exerting inhibition upon motor units when these latter are activated, do not, so long as the motor unit is quiescent, unfold on it any immediately obvious effect at all. Now the reflexes of which it is a question here and for which, when they are concurrent, inhibitory power is suggested, do on the contrary, in the quiescent state of the motor unit, excite contraction of it. Further, in the usual inhibitory reflexes, the afferent arc which inhibits contraction of the given muscle evokes contraction of muscles antagonistic to that. In the present case the afferent arc supposed inhibitory for the given muscle, is for the antagonistic muscles known to be inhibitory, not excitatory. Reflex inhibition if the key to the occlusion is therefore exerted in circumstances departing from those which attach to it usually. Proceeding, however, and in the first place on the supposition that the inhibition is of the kind familiar in reflex inhibition elsewhere, we may apply such inhibition to the case of the occlusions instanced above.

(1) Stating the occlusion result in a general form; afferent arcs A and B are each excitatory of a number of motor units belonging to a given muscle; A, under a given stimulus, evokes a contraction of a certain value, so similarly does B. On evoking B's contraction when A's is already developed, B adds a value less than its own value when acting apart. The assumed inhibition may cause this deficit (i) by reason of A inhibiting some of B's contraction, or (ii) by reason of B inhibiting some of A's contraction, or (iii) by A inhibiting some of B and B some of A. That in no case does the concurrence reduce the contraction below what A, the pre-current reflex, has already developed at the time when the concurrence occurs, would suggest as more probable that A inhibits B and not *vice versa*. Adopting this suggestion, A is the inhibiting reflex and B the reflex suffering inhibitory diminution. But if the sequence of A and B be reversed, so that B becomes the pre-current reflex and A the post-current, deficit still occurs and with retention of the same feature, namely the concurrence never reduces the observed contraction below that of the contraction which B, now the pre-current reflex, has already developed at the time when the concurrence occurs. Therefore, B now appears as the inhibiting reflex and A as the reflex suffering inhibition. In other words, on this showing precurrence determines inhibition. Also, the inhibition is of such a nature that it can merely preclude excitatory activation from arising but cannot suppress it when arisen. These are no features of reflex inhibition as familiar elsewhere.

Reverting therefore to supposition (iii) above, namely, that the deficit is due to A inhibiting some of B and B some of A. On this view, since the deficit of contraction never exceeds the amount of the post-current reflex's independent contraction, the amount of conjoint inhibition must in cases of total occlusion always be exactly countervailed by the post-current reflex adding just so much contraction as equals that which the conjoint inhibition annuls, a feature unexhibited by reflex inhibition elsewhere. Further, in certain instances of total occlusion where the pre-current reflex is activating the sum-total of motor units belonging to the muscle (fig. 13), the post-current reflex can only add its compensatory contraction by activating units within the group belonging to the pre-current reflex, but there the only available units for activation will be those supposed to be freed from activation by the suggested inhibition. The scheme, therefore, invokes at once inhibition and excitation of the same motor units, a self-contradiction.

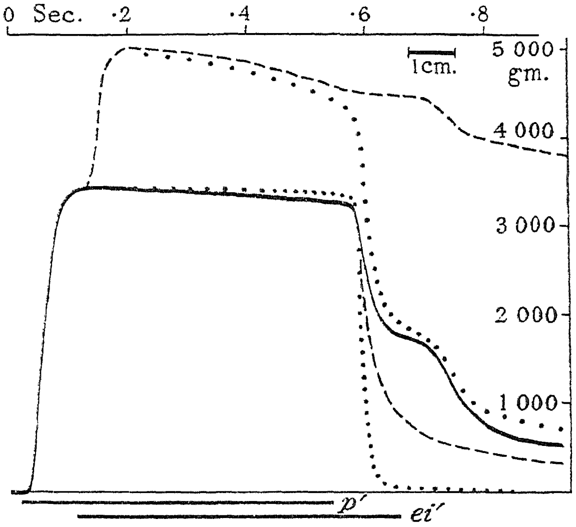

FIG. 13.—Contraction (unbroken line) in response to peroneal stim. (12 cm. coil, 90 per second) during p' followed by stim. (12 cm. coil, 80 per second) of both plantar nerves together during ei'. Upper broken line shows control reflex of plantars in absence of peroneal; lower broken line shows course of control reflex of peroneal alone. The lower dotted line marks the course of the tetanic contraction from maximal motor nerve stim. (12 cm. coil, 90 per second) during p'. The upper dotted line indicates the course of the reflex contraction from $p'+ei'$ had their reflexes simply summed. The rhythms of the concurrent stimuli would yield interference beats of 10 a second, but no corresponding waves break the contraction plateau of the concurrent period, although the occlusion amounts to about 44 per cent. of the resultant contraction. *Semitend.* Magnif. 550.

Moreover, there are wanting from the occlusions certain time-relations and features characteristic of reflex inhibition as familiar elsewhere. Thus, the occlusion reveals no progressive increase indicative of recruitment such as commonly accompanies tetanic inhibition; on the contrary it usually somewhat retrogresses. Again, where, after the concurrence of A and B, A's stimulus is withdrawn and its reflex subsides while B's stimulus still remains in operation, there is on cessation of A no interval before appearance of B's contraction. Such an interval would be expected for B's recovery from inhibition, owing to the usual after-action of inhibition; on the contrary, however, B's contraction follows forthwith. There is no lapse or following re-ascent marking such recovery; nor is there sign of that usual sequel to inhibition, post-inhibitory rebound with augmentation. B's contraction, on cessation of concurrent A, smoothly emerges so to say, and without initial hesitation or delay or augmentation reveals itself unmodified and *pari passu* with subsidence of A's contraction, that subsidence as it were merely unscreening it. In short, B's contraction uninterruptedly replaces that of A and, in so far as the quantitative proportions of the two reflexes allow, substitutes it, displaying smooth continuity with it.

A further difficulty for explanation on the view of reflex inhibition is that the concurrence of the two reflexes in a number of instances where the stimulations of the reflexes are weak appears to cause exaltation (see Section II, § 2) instead of depression of the post-current reflex. This exaltation when it occurs ensues from outset of the concurrence; it is not, therefore, a post-inhibitory rebound. To meet this converse result the supposed inhibition has to be supplemented by a reflex process of facilitation of a lower threshold, a complicated suggestion which, in absence of other confirmation, amounts to a redescription rather than an explanation.

Neither has the inhibitory view any suggestion to offer accounting for the general proportionality between the size of the fractional contraction which the afferent can excite in the test-muscle and the degree of suppression of contraction which, as evidenced by occlusion, it can effect in that muscle. The reflex which gives the largest contraction of the muscle occludes most largely the contraction of that muscle: inhibition as known elsewhere shows no relation of this kind.

(2) Explanation of these occlusions, partial or total, might, however, be sought in a suppression which, though not of the type obtaining for reflex inhibition as met with in the interaction between antagonistic reflexes, is nevertheless "inhibitory" in the same sense that the Wedensky effect by impulse-interference in a nerve-fibre may be called inhibitory. With a Wedensky effect, inhibitory recruitment and after-action, and post-inhibitory rebound

need not be expected among the concomitant events ; the interference view does not envisage them, so that absence of those features from the observed occlusions need not militate against resort to an explanation for which they are alien phenomena. Moreover, the anatomical circumstances, namely, two excitatory paths convergent upon a common one, are such as might be and have been expected to give opportunity for a Wedensky effect. Again, the fact that with the pair of reflexes observed reduction of contraction occurs whichever of the two reflexes is pre-current is in accord with what would be expected in a Wedensky interference. Further, the proportionality, though not a strict one, observable between the amount of contraction effect of a reflex and its occluding power would accord with a requirement of the Wedensky interference.

A feature of the occlusions argues, however, against their explanation by a Wedensky effect. The nerves A and B have in numerous instances been stimulated by shock-series of different rates so that the trains of impulses set up could conceivably interfere at a low rate, *e.g.*, 7 per second at the place of convergence of the two afferents. Such beats should be easily detectable in the contraction response and myogram did the motoneurone discharge wax and wane in consequence of such beats during concurrence of the reflexes. Yet there has been no evidence of such beat-waves in any of the resultant tetani. On the contrary the tetanus-plateau during the concurrent stimulation has been smooth and steady, indeed steadier than that of the component contractions themselves.

Further, decisive against explanation by a Wedensky effect seem the cases of total occlusion where the pre-current reflex activates the sum total of the test muscle. Thus, in example (fig. 13), reflex contraction B, when evoked singly, has a value which is 44 per cent. of that of reflex contraction A. When the two reflexes are concurrent the amount of reflex contraction is the same as A's contraction alone, *i.e.*, 100 ; the deficit shown by the concurrent contraction is therefore 44. To remove 44 by Wedensky interference requires presumably collision of 22 from one source against 22 from the other source. Therefore, to account for the deficit, we may suppose 22 of A and 22 of B suppress each other, leaving thus in B a contraction remainder of 22 which makes up A's contraction-remainder of 78 to the observed contraction 100. But A's 100 units are the 100 composing the total aggregate of the muscle ; therefore, to suppose that the suppression of 22 from A can be countervailed by an increment of 22 additional activated units from B is, since all B's units are of necessity included within A's group (that comprising as it does the sum total of the muscle's units), impossible, because it supposes for certain identical units that at one and the same time they undergo both (1) suppression of their activity, and (2) successful excitation of their activity. And the same argument applies against the assumed inhibition of the whole 44 units, A's and B's, which a Wedensky interference would demand; they cannot be replaced by activated units from elsewhere, because the total aggregate of the muscle being already engaged there is nowhere else whence additional units can come. The difficulty becomes numerically even greater in the case of example (fig. 9B), where the occlusion amounts to two-thirds of the whole concurrent contraction, which latter involved likewise the total aggregate of motor units composing the muscle. The attempted explanation by Wedensky interference results therefore in self-contradiction.

Further, that, as previously mentioned, concurrence of the two reflexes, in instances where the stimulations are weak, appears to result in exaltation instead of diminution of the post-current reflex's contraction offers difficulty for the Wedensky interference as an explanation, since the Wedensky interference, although it might diminish, could never augment the reflex contraction.

(3) Explanation therefore by reflex inhibition either of the kind familiar between competing antagonistic afferents, or of the Wedensky interference type sometimes (21, 14, 1) argued for the central nervous system, seems untenable for these occlusions. We are thrown back upon the other earlier mentioned view, that at some structure down-stream from, and converged upon in common by, the two stimulated afferents, one of them A when activating that common structure tetanically and at sufficient frequence, renders it incapable, as judged by contraction effect, of additional effective activation from another source, *e.g.*, the stimulated afferent B; and this although A's own activation of the common structure can, without let or hindrance, be maintained by A for the time being against its competitor B. The structure at which this result occurs may conceivably be the motor unit to which the two afferents have access in common.

Thus to interpret the occlusion is not to substitute a tacit instead of an explicit appeal to Wedensky interference. Between that latter and the interpretation now submitted there are the following differences. To suppress the post-current reflex by Wedensky interference supposes suppression of a corresponding amount of the pre-current reflex. But as shown above the suppression of the post-current reflex is not effected at expense of the pre-current. Again, to account for the missing amount of contraction, the Wedensky interference demands, besides preclusion of part of the contraction which should otherwise arise, a suppression of part of the contraction which has already arisen. But a point which the foregoing discussion has drawn attention to is that the occlusions yield no evidence of any suppression of already existent contraction but only of prevention of contraction which should arise but has not yet arisen. That feature of occlusion, although it creates difficulty for the Wedensky interference view, creates none for the interpretation now submitted. It is a feature which conforms naturally with recognition that pre-occupancy of the down-stream common unit by an existent rapid tetanic activity, while screening the other afferent's excitation, entails in the occluded unit no suspension of its already existent activity. To obtain a "total" occlusion of the post-current reflex the pre-current reflex must be that one which taken singly provokes the larger contraction; this also finds natural explanation on the view here put forward.

That concurrent stimulation of the two afferent nerves evokes a contraction less than the sum of the contractions observed when the two afferents react apart would thus express the overlap of the two arcs upon motoneurones common to them both. The amount of the deficit of contraction would measure the degree of overlap. That commonly the supervention of B stimulation when A is already operating adds to the already existent contraction, would signify merely that of the motor-units belonging to B some are not included in the field activated by A. That in some cases the supervention of B gives no increment to the contraction already existent under A stimulation, would mean merely that A's stimulation included the whole field of motor units belonging, under the particular strength of stimulus employed, to B. That the supervention of B's stimulation never (except in the instance dealt with above in Section I) causes diminution of the contraction already existent under A, would be but natural because B's action does not counteract that of A, although it may support it. That after cessation of the concurrent stimulation there is no evidence of 'rebound contraction' would be but natural because no true inhibitory process has taken place.

Such an interpretation, moreover, gets support from analysis both of the occlusion and of the release from occlusion. A character of the release is its smooth promptitude. Thus, in example, fig. 13, with subsidence of the pre-current reflex A, the occluded contraction amounting to 44 per cent. of the total capacity of the muscle, emerges from occlusion without initial delay or hesitation. It is so adjusted to the after-discharge of A and to the rate of subsidence of that after-discharge as to make in quantity and time precise allowance for it.

So similarly in examples, figs. 12A, 11B, 10A, 7B. This can scarcely mean else than that the incoming contraction avails itself immediately of those motor units which the outgoing contraction relinquishes, step for step and unit by unit, as these become free from activation by the latter. Abruptness of lapse of the pre-current contraction does not disturb the sequence. Where the pre-current contraction A lapses practically completely, *i.e.*, from all its motor units, almost suddenly, owing to brevity of after-discharge (fig. 14A), the post-current B replaces A's lapsed activation of the motor units by activating them itself so far as its own numerical capacity allows. In the transference of the motor unit from dominance of afferent A as activator to dominance of B as its

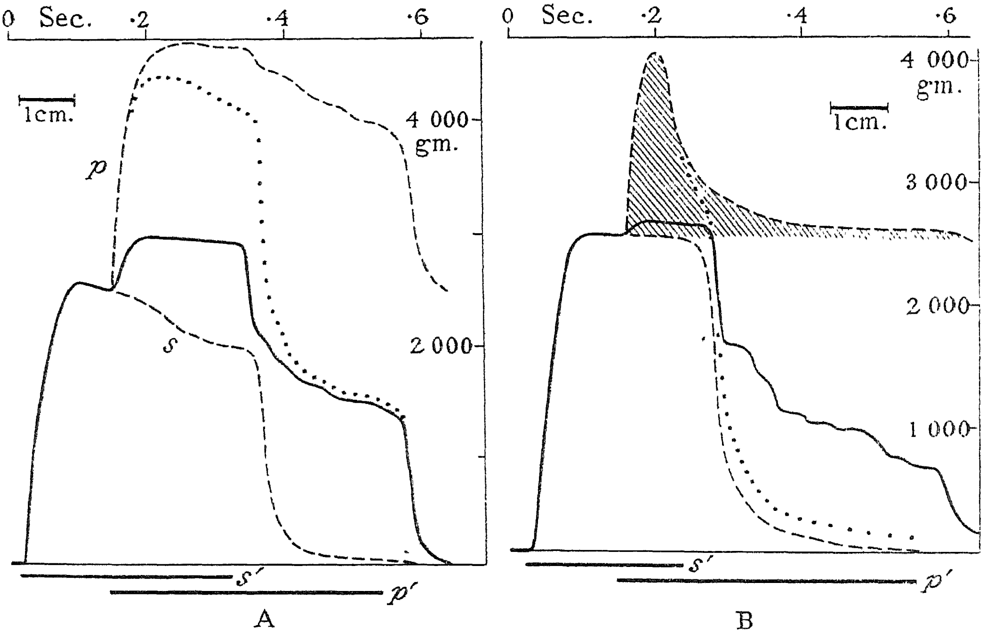

FIG. 14.—A. Contraction by stim. (12 cm. coil) of int, saphen. n. during s' followed by (12 cm. coil) peroneal n. during p'. Unbroken line shows conjoint reflex; upper broken line control observ. from peroneal; lower broken line, control observ. from int. saphen. Dotted line indicates course of contraction had s' and p' simply summed. The post-current reflex after being more than 2/3 occluded emerges practically entire. *Tens. f. fem.* Magnif. 550.

B. Stim. (12 cm. coil) of int. saphen. n. during s' followed by peroneal (13 cm. coil) during p'. Upper broken line and hatched area show control reflex from p' alone; lower broken line, control of s' alone. Dotted line indicates contraction had s' and p' simply summed. Peroneal reflex when alone was "jet-like" and unmaintained: after concurrence with int. saphen. reflex it was better though irregularly maintained. *Tens. f. fem.* Magnif. 550.

activator, the unit reacts as though for it each of the two afferents is equivalent to the other. The replacing of the one for the other exhibits what may be termed "substitution by equivalence."

So rapid and equable is this 'substitution' that not rarely it gives for the time being no hint in the myogram, so that only some subsequent event in the record reveals that it has taken place. Thus, in example fig. 10B, the transfer of activation of some 50 per cent. of the motor units engaged (representing nearly 1000 gm. tension) is accomplished from afferent A to afferent B without trace of any dislocation in the plateau line of contraction of the reflex. The transfer is revealed only on withdrawal of B as inter-current reflex, with confirmation by A's control reflex as separately observed.

The contraction-plateau during the concurrence of the two reflexes is usually steadier than is the plateau of either component reflex alone (figs. 10A, 10B). It would seem as though transient small irregularities due to minor lapses in the one reflex are forthwith repaired and compensated by the substitution-process from the other. The smooth flat plateau which often characterises the increment-contraction of concurrence may have a similar explanation (figs. 4B, 16B).

The contraction of the concurrent period is not only smoother and steadier, but also presents a plateau-height more durable and better upheld than that of either of the component reflexes elicited apart (figs. 10A, 10B).

For the concurrence to leave any marked trace on the reflex which emerges from occlusion is unusual (figs. 5B, 11B, 14A). The emergent reflex exhibits ordinarily neither pronounced reinforcement nor the reverse. Nor does an inter-current reflex with its concomitant occlusion betray marked after-effects (figs. 4B, 7A). Regarded from the aspect of co-ordination the freedom from these consequences may be significant as ensuring that the occluded reflex on emergence from occlusion comes into action undistorted by postponement.

We have, however, met certain instances where a component reflex has emerged from the concurrence and occlusion distinctly modified as compared with its control observed apart from the concurrence. These have been where the post-current reflex contraction has been of the "jet-form" not infrequent among weak flexor spinal reflexes. In them the contraction after a rapidly-reached initial climax wanes rapidly, despite unabated continuance of the nerve stimulation. When a jet-like reflex of this kind is arranged to follow in overlapping sequence a reflex of ample and sustained type, its character is found altered after the concurrence. The jet-like reflex emerges from the concurrence and occlusion considerably improved in staying power (figs. 11A, 14B). Its ability to maintain activation of its motor-units has been reinforced by the concurrence.

Total occlusion and the more rapid declension of the course of a reflex contraction under weaker than under stronger stimulation affords opportunity for more precise determination of the seat of the so-called "fatigue" of a spinal reflex. The whole trend of our observations on occlusion and of the explanation here put forward for it has, like that of many previous observations (18, 13, 26), been against the reference of such "fatigue" to the motor-units themselves; and some of our results appear conclusive on the point. Thus, in fig. 15, with *tensor f. femoris* muscle and stimulation of the int. saph. n. followed by poplit. n. in overlapping sequence, in observation A of the figure the stimulation for both nerves is maximal, and the reflex contraction evoked by int. saph. totally occludes that potentially due from popliteal. On the interpretation offered in this paper, the motor-units activated by int. saph. include all those which popliteal can activate. B of the figure shows the observation repeated, with the difference that the stimulation employed for int. saphen. n. is somewhat weaker, so that, although the reflex contraction reaches practically the same initial height (tension) as before, that height is not maintained but declines from so-called "fatigue" of early onset. The then

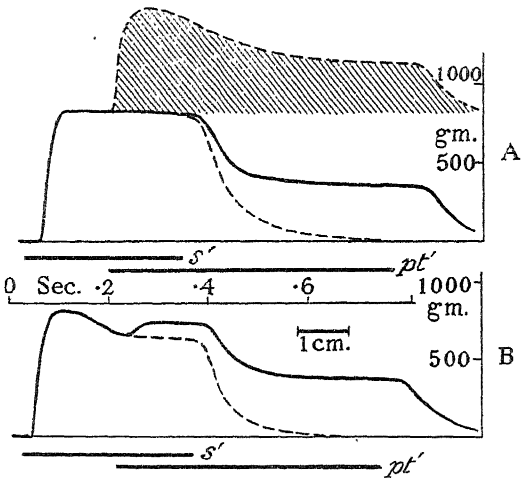

Fig. 15.—Stim. of int. saphen. n. during s' followed by poplit. n. (12 cm. coil) during pt'. In A coil strength for s' was 13 cm.; in B, 15 cm. Upper broken line in A shows control reflex of poplit. alone; lower broken line in A and in B, control reflexes of int. saph. alone. The observations taken together show that the seat of the reflex "fatigue" is not in the "motor units" (see text). *Tens. f. fem.* (ant. portion). Magnif. 390.

ensuent stimulation of popliteal, at the same strength as before, produces in this second instance an increment of contraction. This increment as shown by observation A can only be by activation of motor-units common to popliteal and internal saphenous. It must, therefore, be due to reactivation by popliteal of motor-units whose activation intern. saph. has just previously failed to maintain, namely motor-units which have, a twentieth of a second earlier, lapsed from activity under and despite intern. saph. nerve's excitation. The cause of the failure of the motor-unit's activity can therefore hardly have lain in their own exhaustion seeing that with popliteal stimulation they respond at once.

The phenomenon of occlusion results from the central impingement, mediate or immediate, of two or more afferent nerve-fibres, situated in separate—often widely separate—nerves, upon one and the same motor-unit. It confirms and extends a conclusion reached in our previous paper (9), namely, that of the fibres composing the afferent limb-nerves which reflexly activate the limb's flexor muscles, many, in accordance with the "principle of convergence," activate motor-units which they, so to say, possess in common. This, of course, harmonises with the morphological finding of Cajal (7) and others to the effect that each motoneurone receives its "*boutons terminaux*" from many individual afferent terminals. The extensiveness and range of the occlusions met with by us are in accord with, and would indeed demand, the wealth of convergent terminals upon the individual motoneurones which Cajal's figures depict. Further, the "occlusion" and "equivalent substitution" show that, so far as these processes are concerned, the advent of impulses from other sources need not disturb in the common unit beyond the convergence point, an activation already in operation from a different and prior source. This renders more interpretable in terms of function the morphological finding of Cajal and others that one and the same motoneurone may receive several *boutons terminaux* from one and the same individual afferent terminal fibre.

Occlusion throws some light on the arrangement and constitution of the fractional groups (9) into which the sum-aggregate of the motor-units representing the total muscle is, by the allied reflexes which activate it, subdivided. The observation that "total" occlusion occurs only if one of the concurrent afferents is powerful and so stimulated as to activate a very large and usually heavily preponderant number of the total motor-units shows that apart from exceptional circumstances each afferent's share of representation in the muscle is a fractional group of motor-units not fully identified with that of any one other of the various afferents activating the muscle. In other words the fractional representation which each individual afferent ordinarily has in the muscle is of a pattern peculiar to itself. Even with small fellow nerves so similar in function and anatomical make-up as are the twin afferents medial and lateral of the sural triceps (or of adjacent portions of *quadriceps cruris* (fig. 6)) the partial character of the occlusion they exert on each other (in *semitendinosus*) under even maximal stimulation shows the considerable independence of the respective fractional groups of motor-units proper to them in the muscle. Again, a given afferent (*e.g.*, int. saphen. n. at knee, *cf.* above) with whose reflex on the test-muscle the reflexes of a series of other afferents are in turn made concurrent exhibits a partial overlap of its fractional group with each of them, but a complete overlap of its fractional group with none of them. Such instances exemplify the considerable specificity of pattern of the fractional motor-unit group proper to each afferent, and so to say *representing* it, in the muscle it activates. Each individual afferent therefore when reflexly activating the muscle excites there a contraction of a pattern specific and peculiar to itself. The proprioceptive apparatus of the muscle may distinguish these individual patterns, and in virtue of them supply a proprioceptive basis for reflex " local sign " (24, 27) which as is well-known attaches to, and discriminates between, afferent and afferent. And since each individual afferent nerve activates not one muscle alone but a number, the specificity of the pattern of reflex contraction which each such nerve evokes obtains throughout a multi-muscular field (11). Moreover, that specificity, since it involves a specific set of motor-units, implies also a distinctive set of intraspinal motoneurones. The motoneurones of afferent B least readily occluded by afferent A are those of long after-discharge to B, *e.g.*, fig. 7A, *infra*.

§ 2. *Facilitation.*—The contraction of the concurrent reflexes exceeds the sum of the two contractions evoked by them when acting separately.

It was stated above that when, for examination of the paired afferents which yield an occlusion, weaker stimuli are substituted for stimuli of full strength, not only are the paired contractions weaker but, under concurrence, they exhibit relatively less occlusion. On passing to quite low strengths of stimulus the post-current reflex not rarely shows instead of a deficit of contraction an actual excess as compared with its own isolated control reaction, *i.e.*, with its contraction when not concurrent with the other.

In instance, with *tibialis anticus* muscle (fig. 8) the plantar nerves each maximally stimulated yielded each a contraction of some 1000 gm. On following in overlapping sequence the external yielded a contraction increment of less than 10 per cent. of its own contraction when independent. On the other hand when both were stimulated quite weakly, the internal at 20·5 cm. coil yielding 150 gm. contraction, the external at 17·5 cm. yielding 180 gm., on observing by overlapping sequence their concurrent effect, external as post-current added to internal a contraction increment of 320 gm.

Similarly on *semitendinosus* muscle with peroneal and popliteal (fig. 9B); with two divisions of the nerve of quadriceps extensor (fig. 6), and with peroneal and internal saphenous (fig. 5A).

But such results, although fairly frequent, have not been of regular occurrence; and reflex contractions in response to stimuli not far above threshold are wont to be irregular in size. The control observations as gauge and standard for the size of the interacting concurrent reflexes are for these low values of stimulus much less trustworthy. Nevertheless, accepted at its face value, the contraction excess met under the concurrence of quite weak reflexes argues an interaction which facilitates and augments the reflex response. Such a reaction although in result contradictory to the occlusions of the previous subsection is not so in principle. Camis (8) noted that subliminal stimulations of the two nerves together sometimes gave a slight contraction. Of the motor-units belonging to, *i.e.*, forming the "proper group" (9) of, the afferent nerves of the pre-current reflex some presumably are acted on by it subliminally; and the same must hold likewise for the post-current afferent and its "proper group." These two proper groups consist very largely of motor-units common to both. That a certain proportion of these motor units receiving subliminal excitation from afferent B should coincide with those receiving subliminal excitation from A is therefore not surprising. The facilitation from interaction of B and A would, therefore, be explicable if it might be supposed that the subliminal excitations could summate and by summation become supraliminal for certain of the units.

The above evidence that the pre-current reflex has facilitated the post-current we have met with only when using weak stimuli and weak reflexes. It is to be remembered, however, that the experimental data are merely (1) the reflex as sampled singly, compared with (2) a repetition of the reflex as modified by concurrence. Its modification may be a net result of increment by augmentation and decrement by occlusion. The greater amount of occlusion occurring under stronger stimuli and stronger reflexes, might suffice to obscure the augmentation in the net result.

Section III.—*Occlusion and Inhibition admixed.*

The group of reactions dealt with in Section I showed that between a certain restricted set of the limb-afferents the interaction is truly inhibitory although the inhibition effected is only partial. The inhibitory action of these nerves is accompanied in the *same* muscle by an excitatory action; the limited extent of inhibitory relaxation which they can produce in the muscle may be partly due to the accompanying contraction which they themselves produce and is uninhibitable by them. Thus in fig. 2A the onset of inhibition is preceded by a small peak and the withdrawal of the popliteal stimulation is followed by a further fall in tension, both indicating the presence of the small popliteal increment. These nerves, in respect of the excitation and contraction they produce, interact reflexly by occlusion, even in those muscles in which they exert their inhibition. That the two processes are essentially dissimilar is evident from the long-lasting effect (fig. 2) of true inhibition on the pre-current reflex.

The occlusion and inhibition are both very evident in example fig. 2B where the contraction of *tens. f. fem.* evoked by the inhibito-excitatory afferent popliteal is followed by int. saphen. n.'s contraction which, although dwarfed by inhibition from popliteal, nevertheless engulfs by occlusion (or is occluded by—both amount to the same thing) the remainder of the popliteal contraction. This also happens with the same nerves in reverse sequence (fig. 16B),

Again, with *semitendinosus,* fig. 16A, reacting to full peroneal stimulation, the effect of post-current popliteal is an inhibitory relaxation of part of the peroneal contraction. This might be supposed an unmixed inhibitory reaction; but the control sample of popliteal effect taken without the contraction background exhibits not merely no inhibitory effect (there being no background for revealing that) but a certain amount of contraction. No trace of this contraction appears as obvious increment to the peroneal contraction in the reflex where popliteal is combined with peroneal. It seems submerged in the contraction remainder left as residue despite the inhibition. This is in accord with (1) the fact that in preparations which do not exhibit popliteal inhibition but only popliteal excitation of semitendinosus the peroneal and popliteal contractions liberally interact by occlusion, (2) the fact that popliteal contraction of semitendinosus is not inhibitable by popliteal itself and therefore should belong to the uninhibited residue of the contraction (fig. 16A). Moreover, a particular range of strength of peroneal stimulation can be found at which the peroneal reflex contraction when a full popliteal stimulus is added

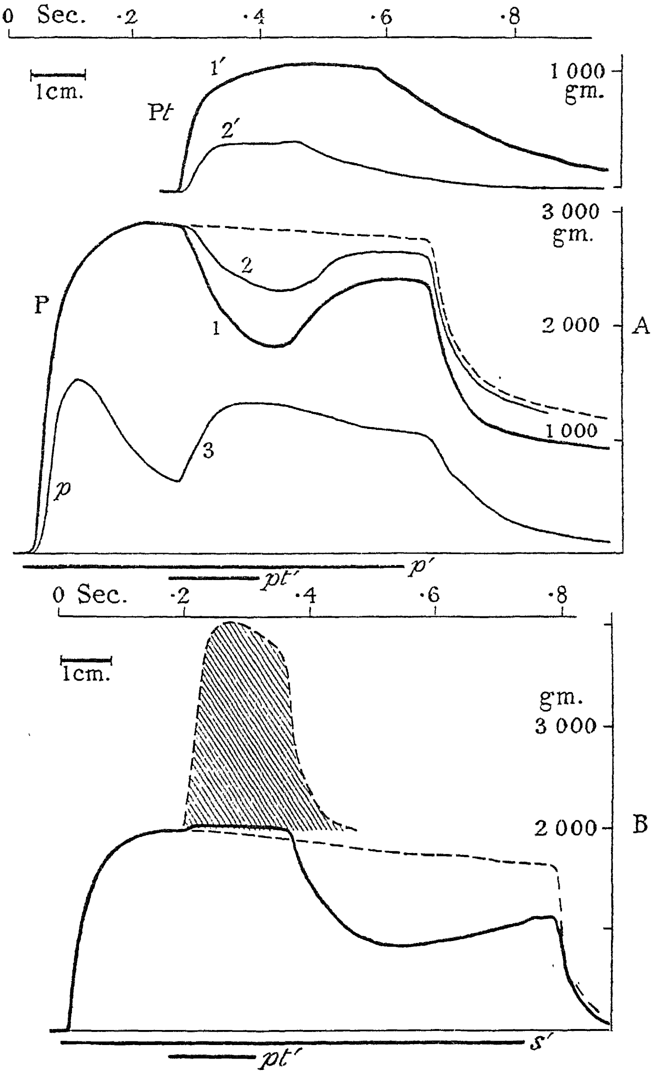

FIG. 16.—A. Stim. of peroneal n. during *p′* followed for a short intercurrent period by popliteal nerve during *pt′*. Peroneal stim. is 13·8 cm. coil for P, 22 cm. coil for *p*. Poplit. stim. is 13·8 cm. for 1 and 1′; 15·5 cm. for 2 and 2′ and for 3. The lines 1′ and 2′ show *pt*'s reflexes when apart from *p′*; line 1, 2, and 3, *pt*'s reflexes when concurrent with *p′*. Broken line continuing P shows control observ. of P's reflex when uncombined with post-current popliteal. *Semitend.* Magnif. 550.

B. Stim. (13 cm. coil, 95 per second) of int. saphen. n. during *s′*, followed for a short inter-current period by popliteal n. (11 cm. coil, 88 per second) during *pt′*. Upper broken line shows control reflex of poplit. by itself; lower broken line, control refl. from int. saphen. alone. Occlusion and inhibition admixed; refl. contraction of poplit. is almost totally occluded, but on withdrawal of poplit. stim. int. saphen. reflex undergoes partial inhibition from popliteal's inhibitory after-action. *Tens. f. fem.* Magnif. 560.

neither undergoes any inhibitory diminution nor exhibits any contraction increment.

In this muscle there were three grades of tension of the pre-current peroneal reflex against which the popliteal effect was differentiated. Below a certain previous tension (fig. 16A) the inter-current popliteal effect was always excitatory, but the increment excited did not carry the tension above this limiting tension (represented by the peak of P in fig. 16A). When the background reflex tension lay between this last-mentioned tension and the tension reached by the floor of the trough of a maximal inhibition the popliteal effect was nil—it was entirely occluded. When peroneal was maximal popliteal could cause varying degrees of inhibition according to its strength—but never an increment of contraction.

Thus the excitatory effect of popliteal upon semitendinosus is occluded by the low threshold units of peroneal. The inhibitory takes effect upon the high threshold units of peroneal. These facts capable of interpretation upon the all or none hypothesis of motor-unit function themselves support the hypothesis.

The case with int. saphenous and popliteal nerves acting upon *tens. fasc. femoris* is similar, but here the popliteal excitatory reflex is itself but little occluded by the reflex from internal saphenous (fig. 16B and fig. 2) when it forms an increment in spite of the presence of inhibition. The inhibition involves in fig. 16B all the units which are activated by internal saphenous except those common to it and the excitatory element of popliteal. In fig. 2 the inhibition does not affect some units proper to intern. saph. itself.

Similarly, in the reflex interaction of popliteal with internal saphenous n. with regard to *tens. f. femoris*, the excitatory effect from popliteal itself seems uninhabitable by popliteal itself. One result of this is that the latent period of the popliteal inhibition is seemingly much prolonged on occasion because the accompanying excitatory effect may mask it for a long initial period (fig. 16B). And the same feature is traceable in the slighter and less regularly obtainable inhibitory effect from peroneal (*v.s.* Section I) shown in fig. 17.

Instances are thus recognisable where the interaction arising from concurrent stimulation of two ipsilateral afferents exhibits as regards certain flexor muscles an admixture of inhibition and occlusion. And this occurs in those cases where one of the paired afferent nerves contains fibres which are, as there are other grounds for supposing, some of them concerned with a flexion reflex of the limb and some of them with an extension reflex of the limb. An afferent nerve is rarely a collection of fibres homogeneous in function. If ‘ occlusion ’ be a co-ordinating element for allied reflexes and inhibition a co-ordinating element for antagonistic reflexes, it is not surprising that, in certain cases, stimulation of one and the same individual afferent nerve, especially if that nerve be large, should evoke both, occasionally even in the field of the same muscle. Rather is it surprising that the interaction evoked between one stimulated afferent and another in the field of the limb should so largely, at least as regards the flexor muscles, be of one class as it is; and should exhibit in so wide a field, unconfused by admixture of inhibition, the characteristic co-ordinating elements of allied reflexes, 'occlusion' and 'substitution by equivalence.'

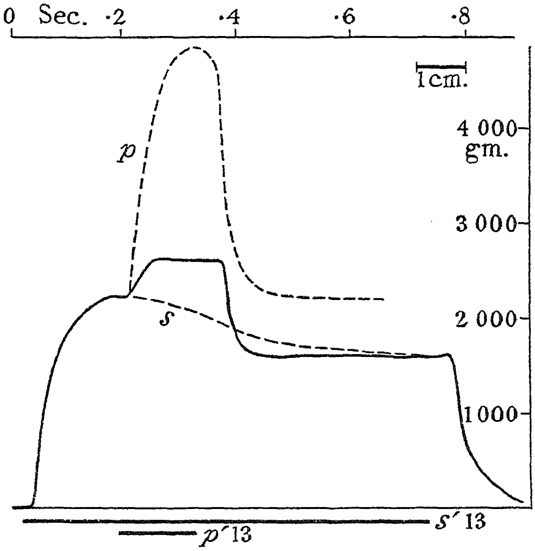

FIG. 17.—Stim. (13 cm. coil, 95 per second) of int. saphen. n. during s' followed for a short inter-current period by peroneal n. (13 cm. coil, 88 per second) during p'. Upper broken line shows course of control reflex of p' alone; lower broken line, control reflex of s' alone. The concurrent period is followed by a steep dip and gradual reascent of tension suggestive (*cf.* fig. 16B) of some "post-concurrence" inhibitory effect. *Tens. f. fem.* Magnif. 550.

SUMMARY.

(1) Examination of the interaction between a pair of allied reflexes in a muscle which they both excite (the pairs of allied reflexes taken being those constituting the ipsilateral flexion-reflex of the spinal hind-limb) finds that the contraction evoked in the muscle by the two afferents acting concurrently is (apart from some limited exceptions, *v. infra*, 12) less than the sum of the contractions yielded by them when not concurrent.

(2) If the missing amount of contraction be referred to as "occluded," the occluded contraction is, with any given pair of afferents, relatively as well as absolutely greater when the reflexes are large than when they are small. In some instances the contraction of the smaller of two concurrent reflexes is occluded totally.

(3) Evidence is given that the occlusion is not referable to reflex inhibition such as characterises the interaction between antagonistic reflexes, nor, granting occurrence of Wedensky interference in the central nervous system, to a process of Wedensky interference.

(4) Examination of the features of the occlusion allows the inference that at some structure, *e.g.*, motor unit, of common approach by the convergent allied afferent arcs (*a*) tetanic activation at sufficient frequence by one of the arcs precludes additional effective activation by the other of them; and that (*b*) activation in process from one arc is unimpaired by concurrent activity of the other arc.

(5) That concurrent stimulation of the two afferent arcs evokes a contraction less than the sum of the contractions observed when the afferents react apart would thus be an expression of the overlap of the two arcs upon motor units which are common to both. The amount of the deficit of contraction would measure the degree of overlap.

(6) Thus interpreted, "occlusion" throws light on the reflex fractionation of a muscle which, as pointed out in our previous paper, is effected by the individual afferent nerves which activate it. Since the reflex of any given afferent when concurrent with that of another allied afferent exhibits, throughout the whole series of individual afferents (apart from exceptional conditions such as involve large nerve-trunks, strychnine, etc.), partial overlap of its "fractional group" of motor-units with that of the other in every case and in no case total overlap with any one other of the whole series, the "fractional group" of motor-units pertaining to each individual afferent cannot for any two afferents be identical, in other words, is specific for each afferent. This amounts to stating that each individual afferent excites in the muscle a reflex contraction which is of a pattern—a spatial configuration—peculiar and specific to that particular afferent nerve. Proprioceptive recognition of this may contribute toward reflex "local sign."

(7) When concurrence of the two reflexes is arranged by overlapping sequence, the occluded contraction, not present during the concurrence, sets in on subsidence of the pre-current reflex, and adds itself to the non-occluded portion of the post-current reflex's contraction.

Cooper, Denny-Brown and Sherrington. *Roy. Soc. Proc., B, vol.* 101, *Pl.* 14.

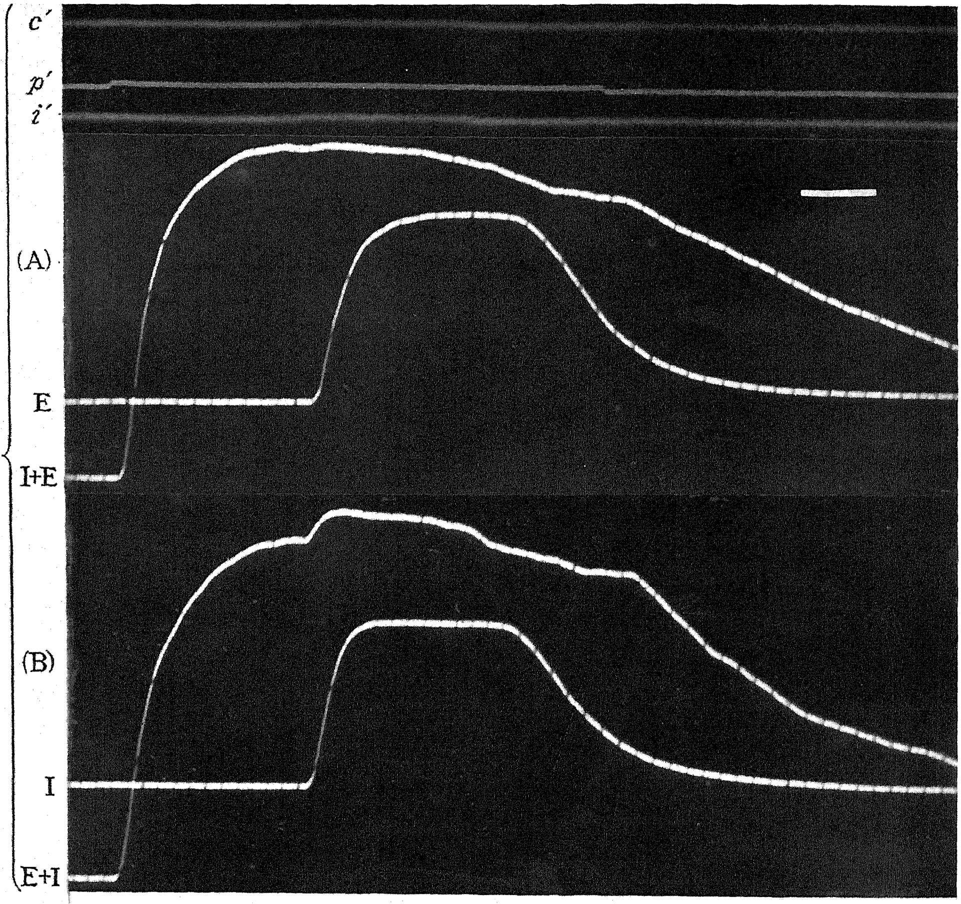

FIG. 1.

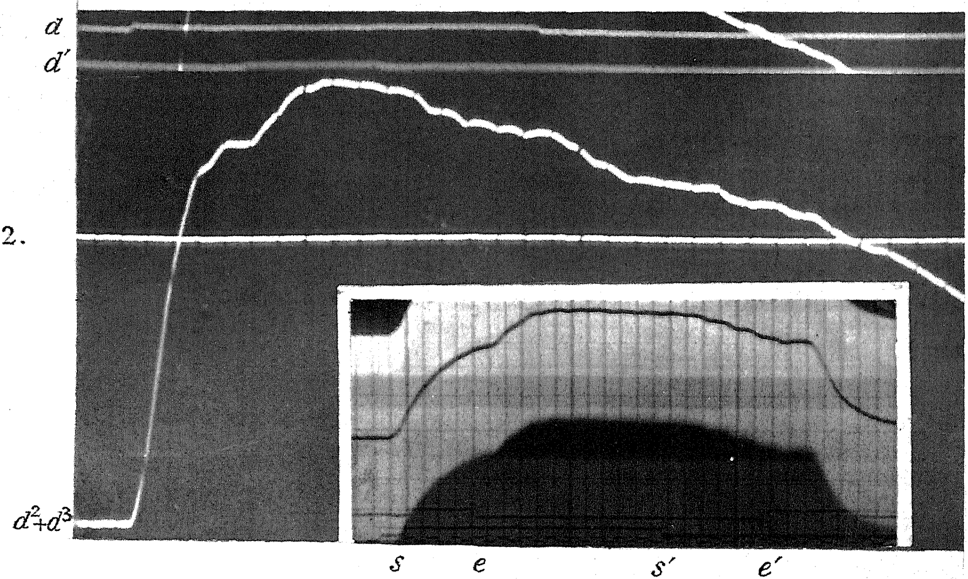

FIG. 2.

(8) The emergence from occlusion occurs without sign of pause, lapse, hestitation or delay and is so adjusted as to fit step for step the subsidence of the pre-current reflex. Thus the elements of the one afferent arc substitute themselves, in regard to the motor units held in common, for those of the other afferent arc, as though the two were equivalent for those motor units.

(9) Contraction under the concurrence of the two reflexes is steadier and more durable than is often that of either reflex acting singly.

(10) The course of the occluded or partially occluded reflex shows after the occlusion usually little or no modification traceable as result from the occlusion. In some instances, however, it has after release exhibited enhanced power of self-maintenance as judged from the control observation taken singly.

(11) With a given pair of afferents which yield occlusion, it is not infrequent when, under weak stimulation, their reflexes are quite small, for concurrence to be accompanied by no evidence of contraction-deficit but to show exaltation of the contraction of the post-current reflex, *i.e.*, a facilitation of the post-current contraction by the pre-current reflex. This suggests that the stimulations exerted on the motor units by the two afferent arcs are so related, in regard to motor units common to the two, that when individually subliminal for activation they can sum to supraliminal value.

(12) In the case of a certain restricted set of the limb afferents and in regard to the hip-flexor, *tensor f. fem.*, and to a less extent the knee flexor *semitendinosus*, in addition to their reflex interaction as " allied " arcs exhibiting co-ordinative " occlusion " and " substitution by equivalence," an interaction exerting inhibition and characteristic of antagonistic arcs has sometimes though not constantly or regularly been found. This seems traceable to an ipsilateral extension reflex whose receptive field is sub-genual and whose afferent fibres are admixed in varying proportions in different individuals with the afferent fibres of the dominant flexion reflex.

DESCRIPTION OF PLATE 14.

Fig. 1.—Contractions of *tib. anticus* elicited as follows: (A) I + E from int. plantar n. (13 cm. coil) during p', followed by ext. plantar n. (16 cm. coil) during i'; almost total occlusion of ext. plantar. E, control reflex of ext. plantar n. (16 cm. coil) alone during c'. Control obs. of I (omitted from plate) conformed with I + E.

(B) E + I from ext. plantar n. (13 cm. coil) during p' followed by int. plantar n. (16 cm. coil) during i'; partial occlusion of int. plantar. I, control reflex of int. plantar n. (16 cm. coil) alone during c'. Control obs. of E (omitted from plate) conformed with E + I, except for the inter-current increment due to I on the plateau: Time in 0·02 sec. Tension ordinates rectilinear, 5 mm. on original = 100 gm. Myograph multiplies tendon movement 205. Short line in top right-hand corner was 1 cm. in length in original.

FIG. 2.—Contraction $d^2 + d^3$ of *semitend.* elicited by dorsal digital n. at 2nd digital cleft during d (13 cm. coil at 45 per second) followed by similar nerve at next digital cleft during d' (13 cm. coil at 45 per second). Control observation (omitted from plate) for d alone developed 950 gm. tension and conformed with contraction $d^2 + d^3$ except for intercurrent increment (130 gm.) due to d'. The control of d' alone developed 1130 gm. tension, *i.e.*, nearly 90 per cent. of d' is occluded by concurrence with d. Stimulus rhythm shows slightly throughout. Myograph multipl. tendon movement 205. Time in 0·02 sec.; tension ordinates rectilinear, 5 mm. + 160 gm.

FIG. 3.—Contraction of isolated posterior part of *tens. f. fem.* elicited by stim. (12 cm. coil, 220 per second) of int. saphen. n. from s to s', followed by ext. cutaneous n. (12 cm. coil, 35 bk. sks. per second) from e to e'. Myograph magnifies tendon movement 390; tension ordinates rectilinear; 16 mm. = 1000 gm. The tension-rise consequent on s exceeds the fall consequent on s'; the tension-rise consequent on e is much less than the fall consequent on e'. The contraction increment from e shows stimulus-rhythm. Time in 0·02 sec.

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The Relation between " Density" of Sperm-Suspension and Fertility as determined by Artificial Insemination of Rabbits.

By Arthur Walton, B.Sc., Ministry of Agriculture Research Scholar.

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(From the Institute of Animal Nutrition, Cambridge.)

Introduction.

Experiments on the physiology of the spermatozoon are in progress in which artificial insemination is used as a test of fertility. One of the variables which enters into the technique is the number of spermatozoa introduced into the vagina of the female. This number will depend upon the " density " of the suspension (*i.e.*, the number of spermatozoa per cubic centimetre) and the volume. The following experiments were undertaken to test the effect of varying the density of the suspension, the volume being constant. Apart from solving a problem of technique it was hoped to gain information on some of the factors which affect fertility or sterility under normal breeding, since it is conceivable that here also the number or density of the ejaculated spermatozoa may play a part.

Material and Methods.

The rabbit was used for these experiments. Most of the stock was kindly provided by Mr. Hammond from his strains of Belgian Hare and Flemish Giant crosses. These animals are denoted in the protocols (Table I) by the initial letters E, C, H, and were identified by numbers tattooed inside the ear, and by ear notches. Other material was bought from reliable sources. Those denoted by the initial letter B were pure-bred Blue Beverens from a single source. Those with X were a mixed stock of local breed. Where colour differences were not sufficient to identify these animals, the number was tattooed inside the ear. Each doe was kept in a separate cage numbered accordingly. The males used in the experiments were surplus stock from Mr. Hammond's strains. For further details of management and information on the reproductive system of the rabbit, reference may be made to Hammond and Marshall (11). The main advantage of the rabbit for experiments with artificial insemination is the absence of an œstrous cycle. Ovulation seldom occurs without copulation, and the does remain " on heat " practically indefinitely and are available for experiment at any time except during pregnancy or pseudo-pregnancy. Pseudo-pregnancy lasts about 17 days, pregnancy about 32 days, and if the young are removed at birth, as in these experiments was invariably the case, the doe is available for experiment a day or two afterwards.

In addition to the protocols of the experiments a card-index of the does was employed, so that a record of the breeding performance of each one was readily available. One difficulty encountered was the occurrence of permanent sterility, which necessitated the periodic testing of the does by normal matings. Usually in each experiment there would be some infertile inseminations. Latterly, if a doe failed to produce young twice (or sometimes thrice) in succession, she was given a normal mating. If two such matings failed she was killed. *Post-mortem* examination usually revealed tubal adhesions to the ovaries. Since this might have been the cause of her failure to produce young in the experiments subsequent to her last litter, the results in these experiments are designated in the protocols by an " (S) " to indicate that failure might have been due to the sterility of the doe. Tubal adhesions were somewhat frequent in the stock subjected to artificial insemination. Lush (19) also found this to be the case. Hammond and Asdell (10) suggest that it may be due to peritonitis caused by penetration of the vagina by the inseminator. It may possibly be due to infection carried from one doe to another by the instrument. Latterly, care has been taken to avoid these possible causes by using an inseminator of large diameter of the pattern figured by Guyer (9) but about half the length, and using a fresh instrument for each doe.

Usually ovulation occurs only when the doe is sexually excited by copulation; hence vasectomised bucks were used in the experiments. The test of fertility was therefore closely analogous to normal mating, the spermatozoa having to find their way to the tops of the Fallopian tubes to fertilise the ova shed 9½ to 10 hours after copulation. To obtain the sperm-suspensions a buck was killed by a blow behind the ears. The abdomen was cut open and the testes, which are usually drawn up into the inguinal canal at death, were removed. The cauda epididymis was then cut up in about 5 c.c. of 0·15M. NaCl (Analytical Reagent). This gave a dense suspension of spermatozoa which, after examination under the microscope for motility, was diluted by the following method:—A series of test-tubes, cleaned (as with other glass ware) by leaving overnight in concentrated sulphuric acid and potassium bichromate, had been washed thoroughly, rinsed with distilled water and allowed to dry. To each was added 18 c.c. of the 0·15M. NaCl solution and the tubes labelled A, B, C, etc.; 2 c.c. of the dense suspension was then added to A and thoroughly mixed by stirring and sucking the liquid up and down the pipette several times; 2 c.c. of A was then added to B, and so on to the end of the series (about 7). In this way dilutions in ascending powers of 10 were obtained. A rough estimate of the density was made by comparison of the opacity of the suspensions. The range required for the experiment was chosen and from each tube 3 c.c. was measured out for each doe to be inseminated.

Insemination was performed with the pipette referred to above. An assistant, seated, held the doe by the hind legs, head downward between his knees. Care was taken that the pipette just passed the brim of the pelvis. The whole 3 c.c. of fluid was injected into the vagina. In order to avoid systematic errors the order in which the various dilutions were used was varied, or a high and a low concentration given alternately. The whole process was carried out at room temperature, and took from 15 to 20 minutes, according to the number of does. As soon after insemination as possible the densities of the sperm-suspensions were estimated with a hæmocytometer. The one used had a depth of 0·020 mm. and Fuchs-Rosenthal ruling. A thick "Allport" cover-glass was employed. A description of this outfit* and the technique is given by Allport (1). "Student" (25) has shown that the distribution of yeast cells on the squares of a hæmocytometer is given by a Poisson Series. This has been tested for spermatozoa and excellent agreement obtained, the statistical methods employed being those given by Fisher (7). It follows, therefore, that the standard error of sampling is equal to $\pm\sqrt{m}$ (where m = number of spermatozoa counted). In order to test the adequacy of the technique of dilution and sampling, several independent counts were made from the same suspensions and from 1/10 dilutions. Errors due to the technique were small in comparison to the large differences between the dilutions used. The procedure adopted was therefore to take 10 independent counts, each consisting of about 100 to 200 spermatozoa. Of the sperm-suspensions actually employed, only those between about 10^5 to

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* Supplied by Hawksley, London.

10^7 per cubic centimetre could be directly estimated, since above or below this number the difficulties of counting were greatly increased. An estimate was therefore made from the most convenient concentration and the others calculated as multiples of this number. Some error is involved in this procedure, but it is fortunate that the convenient concentrations are those that are critical for fertility (see Table I). An unavoidable error also occurred in the process of insemination. Frequently the doe would evacuate some of the fluid, small in amount but impossible to estimate. It is now realised that 3 c.c. of fluid is about the maximum which the doe can conveniently accommodate, and a smaller volume would have been better. It must be noted, however, that even if half the fluid were lost, which is extremely unlikely, this would reduce the number of spermatozoa by only $\frac{1}{2}$, while the difference between any two densities is 1/10.

The procedure of the experiments may be summarised as follows : (1) Preparation of instruments ; (2) Selection of presumably fertile doe ; (3) Copulation with vasectomised bucks ; (4) Preparation of the suspensions ; (5) Insemination ; (6) Estimation of densities ; (7) A month later, registration of the number and sex of the young born.

Experimental Results.

The results are given in Table I. The density of the suspensions (spermatozoa per 3 c.c.) is given at the head of the columns, the actual estimates being grouped between intervals of powers of 10. The column at the left gives the number of the doe in the order inseminated. The number and sex of the offspring of fertile inseminations are shown. " 0 " indicates that the insemination was infertile and that the doe produced young to a subsequent insemination. "0 (S)" indicates that the doe did not subsequently produce young and was probably sterile. The columns are summed at the foot of the table. The percentage fertility is low, even with the denser suspensions. This may be partly due to the technique of artificial insemination, but mainly to the number of experiments which were carried out during the unfavourable months of July, August and September. The sex ratio shows no significant disturbance. There is a slight tendency towards the production of small litters with the less dense suspensions, but the numbers are insufficient to show whether this result is not entirely due to chance. There is a significant decline in percentage fertility beginning at about 10^6 and complete sterility below 10^4.

Table I.—" Density " of Sperm-Suspension and Fertility.
(Explanation in text.)

	Number of spermatozoa per 3 c.c. placed in vagina of doe.								
			10^3	10^4	10^5	10^6	10^7	10^8	10^9
	No. of doe.		♂ : ♀	♂ : ♀	♂ : ♀	♂ : ♀	♂ : ♀	♂ : ♀	
Expt. 4	E 6							0	
February 11, 1925.	E 5							1 : 4	
	E 7						4 : 5		
	E 2						2 : 6		
	E 8						3 : 5		
	E 11						0		
Expt. 9	E 9						1 : 8		
March 18, 1925.	E 5						5 : 4		
	E 8						4 : 4		
	E 11						4 : 7		
	E 2							0	
	C 1							4 : 3	
Expt. 17	E 4					0 (S)			
June 23, 1925.	E 5					1 : 5			
	X 2					0 (S)			
	E 3				0				
	E 6				0				
	E 1				0(S)				
	E 6			0					
	E 14			0					
Expt. 18	E 12						2 : 6		
July 3, 1925.	E 13						5 : 4		
	C 1					0 : 1			
	E 8					0			
	E 17					0			
	E 2				0				
	X 5				0				
	X 4				0(S)				
Expt. 20	X 3				0				
September 24, 1925.	X 1							2 : 0	
	E 16				0				
	E 15							0	
	X 6					5 : 2			
	X 8					0(S)			
Expt. 21	X 2						0(S)		
October 6, 1925.	E 4						0(S)		
	E 1						0(S)		
	E 5					0			
	E 3					1 : 2			
	E 10				0(S)				
	E 14				0(S)				
Expt. 23	E 15						0		
October 31, 1925.	E 16						4 : 8		
	X 3					5 : 6			
	X 6					3 : 4			
	X 1					3 : 5			

Table I—(continued).

			Number of spermatozoa per 3 c.c. placed in vagina of doe.						
			10^3	10^4	10^5	10^6	10^7	10^8	10^9
	No. of doe.		♂ : ♀	♂ : ♀	♂ : ♀	♂ : ♀	♂ : ♀	♂ : ♀	
Expt. 57 May 19, 1926.	H 19						4 : 0		
	B 6		0						
	X 5			1 : 0					
	E 6					2 : 1			
	E 3				5 : 4				
	E 12				4 : 3				
	E 11			0					
	X 7					2 : 2			
	E 5		0						
	B 5						0		
Expt. 58 June 1, 1926.	E 13			0					
	B 9						5 : 6		
	X 3				7 : 4				
	B 8					4 : 2			
	X 6			0					
	H 18				0 : 1				
	E 15					4 : 1			
Expt. 60 July 6, 1926.	B 6		0						
	E 11							6 : 1	
	B 5			0					
	X 15						0		
	B 9				0				
	E 15					0			
	X 3							5 : 7	
	B 8		0						
	B 7						6 : 2		
	X 16			0					
	X 17					0			
	H 18				0				
Expt. 61 August 10, 1926.	X 19		0						
	X 18							0	
	X 7			0					
	B 1						1 : 2		
	B 3				0				
	X 12					0			
	E 6		0						
	E 12							0	
	H 19			0					
	E 5						2 : 2		
	X 5				0				
	E 3					0			
Expt. 62 August 20, 1926	E 15						4 : 2		
	X 13		0						
	B 7					4 : 5			
	B 8		0						
	X 14				0				
	H 18			0					
	X 17						9 : 1		
	X 6		0						
	X 3					2 : 2			
	E 13			0					

Table I—(continued).

<table>
<tr><th rowspan="3"></th><th colspan="9">Number of spermatozoa per 3 c.c. placed in vagina of doe.</th></tr>
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<tr><th>No. of doe.</th><th></th><th>♂ : ♀</th><th>♂ : ♀</th><th>♂ : ♀</th><th>♂ : ♀</th><th>♂ : ♀</th><th>♂ : ♀</th><th></th></tr>
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<tr><td>Total inseminated ....</td><td>........................</td><td></td><td>14</td><td>19</td><td>27</td><td>28</td><td>30</td><td>12</td><td></td></tr>
<tr><td>Fertile ....</td><td>........................</td><td></td><td>0</td><td>1</td><td>6</td><td>14</td><td>19</td><td>7</td><td></td></tr>
<tr><td>Per cent. fertile ....</td><td>........................</td><td></td><td>0</td><td>5·3</td><td>22·2</td><td>50·0</td><td>63·3</td><td>58·4</td><td></td></tr>
<tr><td>Average size of litter</td><td>........................</td><td></td><td>—</td><td>1·0</td><td>5·3</td><td>5·8</td><td>7·7</td><td>6·1</td><td></td></tr>
<tr><td>Sex ratio........................</td><td>........................</td><td></td><td>—</td><td>1 : 0</td><td>19 : 13</td><td>39 : 42</td><td>70 : 77</td><td>24 : 19</td><td></td></tr>
</table>

* Expts. A and B with rabbits belonging to Mr. Hammond.

Table II.—Effect of Excessive Sexual Activity of Male Rabbits.

(Rearranged from data from Lloyd Jones and Hays (18) with calculation of total number of spermatozoa ejaculated.)

Copulations.	1st.	5th.	10th.	15th.	20th.
Volume (c.c.)	0·34	0·21	0·22	0·13	0·10
Density per c.c.	$104{\cdot}5 \times 10^6$	$39{\cdot}5 \times 10^6$	$14{\cdot}7 \times 10^6$	$4{\cdot}2 \times 10^6$	$3{\cdot}5 \times 10^6$
Total number sperm.	$35{\cdot}6 \times 10^6$	$8{\cdot}4 \times 10^6$	$3{\cdot}2 \times 10^6$	$0{\cdot}6 \times 10^6$	$0{\cdot}3 \times 10^6$
Per cent. fertile	72·09	61·11	51·42	41·37	35·55

Interpretation of the Results.

An examination of the individual experiments and the summary shows that a large number of spermatozoa are apparently necessary to ensure fertility, and that there is a significant decline of percentage fertility with the less dense suspensions. More than one interpretation is possible. It may be that the probability of a normal spermatozoon reaching the site of fertilisation within the time that the ova are fertilisable is small. Spermatozoa reach the tops of the Fallopian tubes after 2 to 3 hours. If introduced at the time of ovulation (9½ to 10 hours after a sterile copulation), the chance of fertility is small. Hence, the unfertilised ova are incapable of living longer than about 4 hours (Hammond (11)). In order to effect fertilisation the spermatozoa must be at or near the tops of the Fallopian tubes between the time limits of about 9½ to 14 hours after coitus. To fulfil these conditions the spermatozoa have first to find their way from the vagina to the uterus. Heape (13) suggests that this may be facilitated by a sucking action of the os in conjunction with movements of the uterus. It is not known whether this occurs after artificial insemination or not. Inside the uterus the spermatozoa will be distributed throughout the organ and conveyed either by contraction of the uterus or by their own motility to the upper end. Here they will be brought within range of the entrance of the Fallopian tubes and will swim up towards the ovary. The orientation of spermatozoa against the flow of a liquid was first described by Kraft (15) and has since been confirmed. Some spermatozoa will therefore begin to make their way up the Fallopian tubes shortly after their entrance into the uterus. The greater number will, however, never reach the tubes within the time limits of ovulation and the life of the unfertilised ovum. Hammond (11) describes the distribution of spermatozoa at the time of ovulation (10 hours after coitus). They are readily found in the vagina, cervix and uterus, but considerable search is required to demonstrate their presence in the tubes. It is unlikely that this distribution will be greatly altered in the subsequent 3 hours. The large number of spermatozoa and the diminution in fertility with the less dense suspensions may therefore be an expression of the improbability of any one spermatozoon reaching the fertilisable ovum.

A second possibility is that the spermatozoa are not all capable of surviving the test for fertility. If semen is kept outside the body, under approximately constant conditions, and an estimate made of the percentage spermatozoa motile at given intervals of time, curves are obtained indicative of considerable variation in the survival of motility.* The cause of this variation is not known. It may in part represent differences in composition or structure. Recognisably abnormal forms have been described from time to time (Ballowitz (2), Williams and Savage (26)). While these are undoubtedly extreme divergences, it is not unreasonable to suppose that slight deviations from the normal, sufficient to affect viability, occur in the processes of formation and development. The age of the spermatozoa may also be a cause of variability. Many authors, *e.g.*, Redenz (22), Benoît (3), regard a sojourn in the epididymis as necessary for the full development of the spermatozoa, and it is possible that many of those taken from the epididymis have been formed too recently to be completely mature. The converse is also possible, namely, that many of the spermatozoa may be over-mature and in process of degeneration. This seems less likely, however, as Hammond and Asdell (10) have shown that the spermatozoa in the epididymis retain their fertilising capacity for over three weeks.

A third point to be considered in interpreting the results is the toxicity of the medium. Toxicity is used here in the widest sense of the word and implies any reaction which directly or indirectly affects the survival of fertilising capacity. Hammond and Asdell, in the paper mentioned above, show that the conditions surrounding the spermatozoon in the epididymis are favourable to its preservation. This is also the view of Redenz (22), who has studied the survival of motility and who concludes that one factor involved is the low concentration of electrolytes. According to this author also, the medium contains proteins and is well buffered to about neutrality. Dilution with NaCl will therefore introduce many presumably undesirable changes. In considering the possible results of toxicity, a distinction may be drawn between a general toxicity affecting the spermatozoa and a differential toxicity acting more vigorously in the suspensions of greater dilution. General toxicity is not peculiar to artificial insemination. If the conditions present in the epididymis be taken as a standard for comparison, Hammond and Asdell (10) have shown that the female tract is relatively toxic since here spermatozoa will not retain their fertility for longer than 36 hours. There is also some evidence that the secretions of the vagina are relatively unfavourable to the preservation of the spermatozoa. Toxicity would account for the large number of spermatozoa required to ensure fertility, and a differential response, due to the variability of the spermatozoa, as described above, would account for the effect of dilution. Differential toxicity of the various dilutions is also possible. Cohn (4) explains the greater survival of dense suspensions of *Arbacia* spermatozoa as due to an accumulation of CO_2 raising the hydrogen-ion concentration of the sea-water. Consequently, the activity of the spermatozoon is inhibited and the limited store of energy present in the spermatozoon is preserved. This hypothesis can possibly be extended to any unfavourable reaction, the products of which would accumulate in the denser suspensions and inhibit further reaction. Differential toxicity of the medium (saline solution and the secretions of the female genital tract) may therefore play a part in determining the results of the experiments.

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* **Experiments in collaboration with Mr. Hammond are in progress.**

Discussion.

The experiments were planned to test a problem of technique, and undoubtedly show that control of density is necessary when the relative fertility of various sperm-suspensions is under consideration. The experiments do not give quantitative data which will distinguish the effects due to (*a*) the probability against a meeting of the spermatozoa and ovum in the female tract, (*b*) the variability of the spermatozoa as regards fertilising capacity, or (*c*) the effects due to differential toxicity. Quantitative data on these points are essential before the results of the experiments can be applied with any certainty to normal breeding. However, a comparison of the results with the data from Lloyd Jones and Hays (18) in Table II shows that as far as the number of spermatozoa is concerned the results obtained are not markedly different from those which might arise normally. (For further discussion see below.) It may therefore be justifiable to discuss the bearing of these experiments on problems of fertility and sterility.

In 1780 Spallanzani (24) made quantitative experiments on the amount of seminal fluid necessary to fertilise the eggs of amphibia. He found that the seminal fluid was active in extreme dilution, but that the percentage eggs fertilised diminished successively. Unfortunately, he failed to recognise the essential nature of the spermatozoon. In 1824 Prévost and Dumas (21) brought evidence that the spermatozoa were the essential constituents of the semen, and they repeated Spallanzani's experiments with greater precision, making quantitative determinations of the numbers of spermatozoa and eggs. For example, in one trial they estimated that 225 spermatozoa fertilised 61 eggs out of a batch of 380, of which 254 were probably fertilisable. Other trials gave very variable results, and they summarise their findings by the statement: "On pourrait être tenté de supputer, d'après nos données, *la probabilité de la fécondation*,* mais il est aisé de sentir qu'il faudrait des milliers de résultats, avant d'obtenir une valeur approchée." Since that time research has been mainly concerned with elucidating the mechanism of fertilisation, and it has been clearly established that only one spermatozoon is necessary to fertilise each egg. Emphasis of this point has perhaps led to a neglect of the quantitative aspect of fertility, namely, that while *fertilisation* is dependent upon one spermatozoon alone, *fertility* or the *probability of fertilisation* depends upon a number, large or small, according to the viability of the spermatozoon, the number and fertilisability of the ova, and the conditions, favourable or otherwise, to an approximation of the gametes. The importance of the density of the sperm-suspension has been well established in experiments with marine and other organisms having external fertilisation (Lillie (16) (17), Fuchs (8), Cohn (4)). This work is mainly concerned with the mechanism of fertilisation or the physiology of the spermatozoon or ovum and is less concerned with the fertility of the organism. Conditions are rather different in animals having internal fertilisation, where the fertility of the animal rather than the mechanism of fertilisation is under consideration. A few typical examples may be given where the density or number of the spermatozoa may have influenced the results of the experiments. The problem is of practical importance in animal husbandry, since it implies that the male may have a greater influence on fertility than has generally been admitted.

Lloyd Jones and Hays (18) found a decrease of fertility following excessive sexual activity of male rabbits. The males were allowed to copulate as many as 20 times within the space of 3 to 4 hours. Tests of fertility and estimates of the volume of the ejaculate, density, rate of movement and survival of motility of the spermatozoa were made at intervals. The data are summarised in Table II and a calculation of the total number of spermatozoa is added. Owing to their method of obtaining semen from the vagina of the living animal, the volume and therefore the total number is probably much underestimated, especially towards the end of the series of copulations. The authors are inclined to emphasise the differences in motility and physical properties of the semen, but it is at least possible that the diminution in fertility is due to the reduction in the total number of spermatozoa. Marshall (20), Crew (6), Hammond and Asdell (10) record experiments where selective fertilisation apparently followed double matings. A difference in the number of spermatozoa ejaculated would sufficiently account for these results, although a difference in vitality of the two sorts of spermatozoa may also have played a part.

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* Italics of author.

Ivanoff (14) has shown a higher percentage foaling return from mares artificially inseminated than from normal matings. This may be partly due to close supervision and a greater frequency of insemination, but the semen is introduced directly into the uterus and it is possible that this ensures a larger number of spermatozoa available for fertilisation. Ivanoff mentions also that stallions differ considerably in their percentage foaling returns (see also Heape (12)). Sanders (23) has confirmed this by statistical analysis, and has also shown that there is a distinct decline of fertility with old age. Differences in spermatogenic activity and the number of spermatozoa ejaculated may be a possible cause. Williams and Savage (26) find infertility of bulls correlated with the appearance of a high proportion of abnormal spermatozoa. Many of their cases can be regarded as indicative of considerable pathological disorder, but a high proportion of abnormal spermatozoa may indicate a small number of normal ones and hence a diminution in the probability of fertilisation. These examples and the experimental evidence of this paper appear to justify the hypothesis that the fertility of a mating, at least as far as the male is concerned, is essentially a statistical problem and measurable in terms of probability.

Summary.

1. Experiments on artificial insemination of rabbits show that fertility is influenced by the density or number of spermatozoa introduced into the vagina of the female.

2. The percentage fertility is reduced where the estimated number of spermatozoa is less than about 10^6 per c.c. Sterility occurs where the number is below 10^4 per 3 c.c.

3. Three factors probably play a part in determining the results, but are not subject to separate quantitative estimation: (*a*) The probability of any one spermatozoon reaching the fertilisable ovum is small; (*b*) the spermatozoa are variable and not all capable of fertilisation; (*c*) toxicity may act differentially on sperm-suspensions of varying density.

4. The bearing of these experiments on the problem of fertility is discussed,

and the quantitative basis of fertility, as far as the influence of the male is concerned, is advanced as a working hypothesis for future enquiry.

Acknowledgments.

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Changes in the Ovary of the Mouse following Exposure to X-Rays.—Part III. Irradiation of the Non-Parous Adult.

By F. W. Rogers Brambell (Fellow of the International Education Board) and A. S. Parkes (Beit Memorial Research Fellow).

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(From the Department of Anatomy (Embryology and Histology), and the Department of Physiology and Biochemistry, University College, London.)

[Plates 15–16.]

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I.—Introduction.

The ovaries of a series of mice X-rayed at weaning time (3 weeks old) were described in the first part (1) of this series of papers, and of a series X-rayed at or before birth in the second part (2). The œstrous cycles of these animals were dealt with elsewhere (Parkes (4, 5)). In almost all animals normal œstrous cycles were maintained, although the ovaries were devoid of follicles and, in most cases, of corpora lutea. Histological study led us to conclude that following irradiation :—

(1) All the oocytes degenerated completely, and that the cells of the membrana granulosa, and of the theca interna, in follicles where it was differentiated, subsequently followed their example. In some cases the follicles did not degenerate, but grew and formed corpora lutea atretica. These persisted but had no effect on the cycle.

(2) The germinal epithelium proliferated cords of cells, which pressed the remnants of the old stroma into the medullary region. These cords formed the major part of the irradiated gonad and were probably the chief source of œstrin.

(3) The germinal epithelium in some cases proliferated a second lot of cords, which resembled "anovular" follicles described by some authors. They also resembled the so-called "tubules" of some free-martin testes

and structures described in the rabbit ovary by Hammond (3) as "spermatic tubules." These cords were shown to have no influence on the œstrous cycle.

(4) The animals X-rayed at birth were divided into three groups: *Group I*—The cells of the first proliferation were small, shrunken and vacuolated. The œstrous cycles were irregular and in some cases prolonged vaginal cornification occurred. *Group II*—The cells of the first proliferation were large, healthy and glandular in character, and somewhat resembled luteal tissue. The œstrous cycles were normal and regular. *Group III*—The cells of the first proliferation very closely resembled luteal tissue. The œstrous cycle was absent or had ceased for at least 36 days previous to being killed.

It was pointed out in these papers that the cords of the first and second proliferations, like the follicular elements, were derived from the peritoneal epithelium. Further, in some cases the cords of the first proliferation produced tissue closely resembling true luteal tissue, normally derived from atretic or discharged follicles.

The present series of experiments was carried out with a view to corroborating these results on the adult animal. It was thought that a hiatus might occur in the œstrous cycle following the degeneration of the old ovarian elements and before the new tissues became functional. No such hiatus has, however, been demonstrated, and it was found on histological examination that no post-irradiation proliferation, similar to that in the immature animals previously described (1, 2), occurred. The elements composing the ovarian cortex at the time of irradiation gave origin to all the tissues of the sterilised ovary.

The œstrous cycles of this series of animals are dealt with elsewhere (6).

The previous literature is summarised in the earlier papers (1, 4) and need not be dealt with again.

Our thanks are again due to Prof. J. P. Hill, F.R.S., and Dr. F. H. A. Marshall, for criticism and advice.

The animals were drawn from the colony maintained with the aid of grants, firstly, from the Government Grants Committee of the Royal Society, and afterwards from the Medical Research Council.

II.—Material and Technique.

The material employed in this paper consists of both ovaries of 44 adult female mice X-rayed at various stages of the œstrous cycle. In all animals a number of œstrous periods were observed before irradiation and any showing irregularities in the cycle were discarded from the series. The technique of the detection of the œstrous cycle and of irradiation is described in the paper dealing with the œstrous cycles of these animals, but it may be emphasised here that, contrary to previous practice, the irradiation (¼ B tint Levi's pastilles) was given in one dose in order that a more accurate time relation of the reaction could be secured. Irradiation was performed as far as possible during diœstrus, but owing to the necessity for irradiating a number at one time, some of the animals were in œstrus when exposed. After irradiation certain of the animals were mated with normal males, but the majority were left for observation of the unmated cycle.

Table I gives a list of the animals employed, together with the time of killing after irradiation and after the last œstrus, and particulars of whether or not the animals were sterile and mated after irradiation.

It will be seen by a glance at this table that the series of animals killed at successive stages after irradiation was ample. The criterion of sterility employed by us in this, as in previous work, was the absence of oocytes from the ovaries. If either ovary contained even a single follicle enclosing a degenerating oocyte it was considered, for the purposes of this work, to be fertile. Only when the oocytes had ceased to exist as cells, and were at most represented by the crumpled, amorphous remnants of the zona pellucida, was an animal considered to be completely sterilised.

It will be seen from Table I that none of the 15 animals killed during the first five weeks after irradiation were sterilised. Twenty-two of the 29 (or 75 per cent.) animals killed more than five weeks after irradiation were sterilised.

No correlation was found to exist between the stage of the œstrous cycle when the animals were irradiated and the histological condition of the ovaries.

The ovaries were fixed in Bouin's fluid immediately after the animals were killed. Complete serial sections were made of each ovary and were stained with Ehrlich's hæmatoxylin and eosin or by Pasini's method.

III.—DESCRIPTION.

The germinal epithelium.—No proliferation of cells from the germinal epithelium was observed in any of the animals considered in this paper. In all the ovaries, ranging from two to 73 days after irradiation, the germinal epithelium was cubical and thin, and epithelial cells were not being budded off from its under surface (Plate 16, figs. 1 and 3). The evidence of this was uniformly clear and indisputable.

Table I.

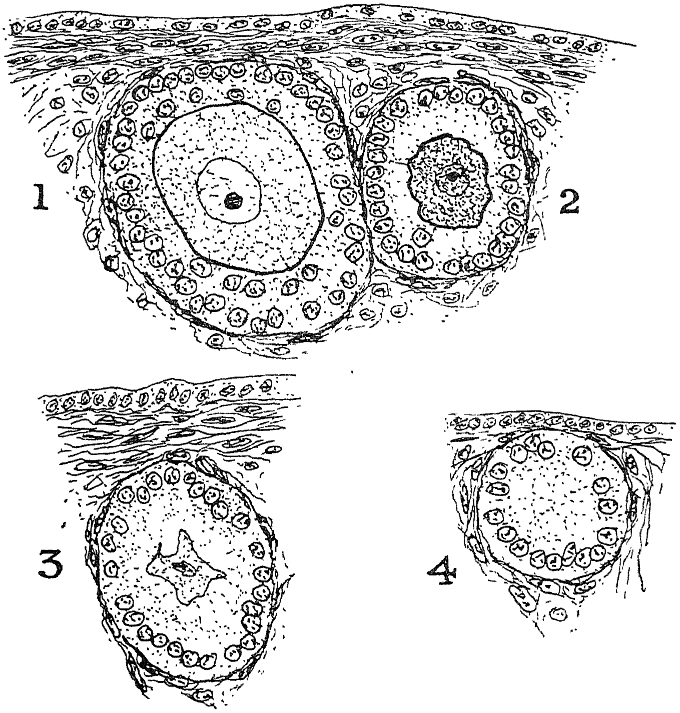
AX.	Days after irradiation when killed.	Days after last œstrous period.	Sterile.	Mated.	Remarks.
1	73	2	+	—	
2	73	3	+	—	
3	73	3	+	—	
7	73	2	+	—	
12	73	1	—	—	
30	67	3	+	+	Pregnant 6 days after irradiation. Produced and reared litter of 7.
31	67	0	—	—	
33	67	11	+	—	
34	67	Cornified for 35	—	—	Normal cycles previous to cornification.
37	67	2	+	—	
39	67	2	—	—	
41	67	11	+	—	
44	67	13	+	—	
45	67	1	+	—	
19	66	0	+	—	
20	66	0	+	—	
23	66	29	+	—	
24	66	5	—	—	
9	64	18	+	—	
46	55	Cornified for 9	+	—	20 days diœstrus before cornification.
47	55	18	+	—	
48	55	17	—	—	
49	55	0	+	—	
50	55	3	—	—	
51	55	24	+	—	
53	55	4	+	—	
16	48	8	+	—	
17	48	6	+	—	Three ovaries developed.
64	40	6	+	+	
54	33	20	—	—	
66	30	2	—	+	
65	30	23	—	+	
59	30	0	—	+	
58	30	27	—	+	
57	30	0	—	+	
61	25	5	—	+	
6	24	0	—	—	
52	20	8	—	—	
55	18	14	—	+	
25	16	12 days pregnant	—	+	Pregnant 4 days after irradiation. Uterus contained 9 fœtuses.
60	14	5	—	+	
62	10	9	—	+	
13	3	0	—	—	Corpora lutea absent.
38	2	1	—	—	
44	—	—	22	12	

The tunica albuginea.—The absence of epithelial proliferation was borne out by the condition of the tunica albuginea. It remained at all stages continuous and well developed beneath the germinal epithelium (Plate 16, figs. 1 to 3). It consisted in the adult of a distinct sheath of fibroblasts several cells thick. In the irradiated animals it remained in this condition, becoming rather thicker and more fibrous if anything, and no epithelial cords were observed to perforate it. It is clear, therefore, that the germinal epithelium did not contribute, after irradiation when adult, to the tissues constituting the sterilised ovary, as it did in the immature animal. In the sterilised animals of the present series these tissues must have been derived entirely from the elements already composing the cortex at the time of irradiation.

The oocytes.—Irradiation resulted in the immediate disappearance of all the small oocytes. Two days after irradiation (AX 38) no small oocytes were present (Plate 15, fig. 5). The majority must have degenerated but some may have rapidly completed the growth stage. All the oocytes present at this and subsequent periods after irradiation were full grown and invested in follicles with from one to many layers of membrana granulosa cells, and in the latter a large antrum. The majority of the oocytes two days after irradiation exhibit signs of degeneration. Some of them exhibited maturation spindles and polar-body formation. Others had ruptured and were being absorbed by leucocytes which had invaded the follicle. The follicle cells were never observed to take on a leucocytic function, nor to assist in the resorption of the oocyte. The zona pellucida was very persistent, as previously described (1), and remained as a crumpled amorphous mass in a small cavity long after the oocyte had degenerated and the follicle, as such, had ceased to exist. No neo-formation of oocytes occurred after irradiation in any case.

The follicles.—Structures identical with the " cords of the second proliferation " described in the earlier papers were present in all the animals of the AX series. In these they were not derived directly from the germinal epithelium and were always found in the periphery but within the tunica albuginea. Their presence even in AX 38, the first of the series killed only two days after irradiation, suggests that they were derived from small follicles in which the oocytes had degenerated. This is confirmed by the presence of the remains of the oocytes in the centre in some cases. Text-figs. 1 to 4 represent the method of their formation.

The fully formed structures were described in detail in the earlier papers and are figured in AX 38 in Plate 16, fig. 1. Since, in the adult animals, they are derived directly from small follicles in which the oocyte has degenerated they will be termed in future "anovular follicles." This term was avoided in the earlier papers as they were derived some time after irradiation directly from



Figs. 1–4.

the germinal epithelium and never contained an oocyte. These anovular follicles appeared to grow to some extent after the degeneration of the oocyte, and mitoses were observed sometimes in the membrana granulosa cells composing them. The growth was slight, however, and they seldom exceeded a diameter of about 80 μ. They appeared to persist indefinitely and were as numerous in the animals killed 73 days after irradiation as in the earlier stages.

Some of the medium-sized follicles, with the membrana granulosa from one to several cells thick, appeared to degenerate completely, and to be ultimately represented only by a small cavity containing the remnants of the zona pellucida. The follicular degeneration is similar to that described in the animals X-rayed at weaning time (1). The majority of the medium-sized and large follicles did not degenerate, however, but their elements grew (Plate 16, fig. 1) and persisted in the sterilised ovary. In these follicles the theca interna enlarged by division and growth of the cells. Mitoses were frequent in both the theca interna and the membrana granulosa at the time when the oocyte was degenerating. Finally, the membrana granulosa filled the entire cavity of the follicle. At this stage the line of demarcation between the membrana granulosa and the theca interna was indistinct, and the individual cells of each closely resembled those of the other. The whole, at first surrounded by the definite sheath of the theca externa, increased in size and became merged with the surrounding tissue to form a mass of large glandular vacuolated cells, closely resembling the so-called interstitial cells of other mammals. Both theca interna and membrana granulosa cells appeared to participate in this process; both hypertrophied and exhibited many mitoses, but their elements became so similar that they could not be distinguished in the later stages. This process was foreshadowed in two or three of the mice irradiated at weaning time, in which the follicular cells were observed to have persisted and formed a zone around the cavity containing the remnants of the zona pellucida of the oocyte.

Only a very few of the large follicles formed corpora lutea atretica, unlike those in the immature animals in which this form of atresia was the rule rather than the exception. Occasionally a large follicle became cystic, as described previously (1, 2). Small cysts were found in AX 13, 16 and 33.

The corpora lutea.—Corpora lutea are present in all the animals, with the exception of AX 13, which will be discussed later, and possibly of three or four of the animals kept for a long time after irradiation in which the entire tissues of the ovary were so luteal-like that it was impossible to be sure of the existence of true luteal tissue. The corpora lutea were present in those killed soon after irradiation and in those kept for a long period after in approximately equal numbers. They did not contain a central blood clot surrounding a cavity containing the remains of the zona pellucida, and had every appearance of being, in the vast majority of cases at all events, corpora lutea vera. It would appear that most were already present in the ovaries at the time of irradiation, but a few may have been formed from follicles which ruptured soon after irradiation. AX 25 and 30 became pregnant four and six days after irradiation respectively, which indicated that ovulation, presumably accompanied by the formation of corpora lutea, may occur shortly after irradiation. The occurrence of corpora lutea in the long-period cases supports our previous finding that they persist indefinitely in the sterilised ovary. Some of these persistent corpora lutea appeared healthy and showed scarcely any signs of retrogression. Others were obviously retrogressing and the luteal cells were much vacuolated.

The interfollicular tissue.—The interfollicular tissue hypertrophied after irradiation, and, together with the follicular derivatives, which were indistinguishable from it, formed thick finger-shaped cords and lobes between the corpora lutea. This tissue formed the main part of the sterilised ovary and, together with the corpora lutea and the anovular follicles around the periphery, constituted the entire organ. It closely resembled in histological characters the cords of the first proliferation, derived from the germinal epithelium, in the animals X-rayed before puberty. In some cases the cells of this tissue remained small and vacuolated, and the tissue resembled that of Group I of the BX series. AX 34, 45 and 49 were good examples of this type, and one of these, AX 34 (Plate 15, fig. 4), had exhibited prolonged vaginal cornification, like several of the Group I BX animals. AX 46, the other animal exhibiting prolonged cornification, did not conform to this type, the cells being larger and less vacuolated, like the majority of cases.

In a number of cases this glandular tissue resembled luteal tissue to a greater or less degree, like the cases in Groups I and II of the BX mice. AX 23 and 47 (Plate 15, fig. 2 ; Plate 16, fig. 3) were among the most extreme cases of this sort, and in them the last œstrous periods were respectively 29 and 18 days previous to killing. Other examples, however, exhibited a regular cycle. On the whole it was impossible to classify the AX mice in clearly defined groups according to the characters of the post-irradiation tissue, as was done with the BX series, but the same types were present, although less defined.

The medulla, etc.—The medulla remained practically unchanged after irradiation, and consisted as in the normal animal of loose fibrous connective tissue containing many blood vessels. The hilum and mesovarium were also normal and the vestiges of the rete appeared unaltered. The vascular supply of the irradiated ovaries was ample and unimpaired. No epithelial canals, similar to those described in the M and BX series, were developed in the AX animals.

The ovaries varied considerably in size, but were, on the whole, not abnormally small. This was probably due to the persistence of the corpora lutea, which form so large a part of the normal ovary.

Special cases.—AX 34 and 46 showed prolonged vaginal cornification for 35 and 9 days prior to killing respectively. The œstrous cycle of AX 34 was normal previously, but AX 46 was in diœstrus for 20 days before cornification set in. AX 34 was fertile but AX 46 was sterile. The ovaries of both contained corpora lutea, but the cells of the post-irradiation tissue of AX 34 were much vacuolated (Plate 15, fig. 4), while in AX 46 they were larger and less vacuolated, like the majority of animals. Therefore AX 34 resembled histologically the BX Group I animals, several of which also exhibited prolonged cornification.

AX 13 had no traces of corpora lutea, although it was killed three days after irradiation. Luteal-like tissue was entirely absent and no corpora lutea can have been present in the ovary (Plate 15, fig. 1) at the time of irradiation, which was during the last day of the diœstrous period.

AX 17 was unique in possessing three distinct ovaries, two close together on the right side and one on the left. The two on the right side were both rather smaller than the left ovary. They were both complete and each had a separate hilum, but they were situated close together in relation to the one Fallopian tube. Their exact anatomical relations could not be made out as they were not discovered until they were partly dissected out. This is the only case which has come within our personal experience of the presence of an accessory ovary in the mouse.

AX 25 and 30 were mated after irradiation, and became pregnant following copulation on the fourth and sixth days after irradiation respectively. AX 25 was killed when 12 days pregnant and contained nine fœtuses, seven in one cornu and two in the other. AX 30 gave birth to and reared a litter of seven. The ovaries of AX 25 were not sterilised. This would be expected as it was killed 16 days after irradiation. AX 30 showed a normal œstrous cycle after the end of lactation. It was killed 67 days after irradiation and the ovaries (Plate 15, fig. 3) were found to have been completely sterilised.

IV.—DISCUSSION.

(*a*) *Comparison with Animals X-rayed before Puberty.*

The ovaries of the present series of animals differ in one essential respect from those of the animals described previously, which were irradiated at three weeks old, or at or before birth. The tissues composing the sterilised ovaries in the AX animals were derived entirely from those already forming the cortex at the time of irradiation. The tissues composing the sterilised ovaries of the M and BX series were formed almost entirely by proliferations from the germinal epithelium after irradiation, with the exception of the corpora lutea atretica. This fundamental difference in the effects of X-rays on the mature and immature ovaries is very remarkable. It was suggested in Part II (2) that the epithelial proliferations following irradiation in the immature ovaries might correspond to the normal epithelial proliferations of the developing gonad. On this view the irradiation of the young animal would stimulate the epithelial proliferation in the ovary, which normally continues from the formation of the gonad until near sexual maturity. On the other hand, the irradiation of the adult, in which the germinal epithelium had ceased proliferating, would fail to stimulate it to renewed activity, and the elements already composing the cortex would give origin to the tissue of the sterilised ovary.

The tissues composing the sterilised ovaries of the present series of animals and of those described previously completely resemble each other, despite the apparent difference in origin, as a comparison of the plates will show. All the chief types of tissue, exhibited by the first proliferation in the BX and M mice were also represented in the AX series. Moreover, the sterilised ovaries of all three series maintained the œstrous cycles.

The tissue of the sterilised AX mice was derived from the follicular and interfollicular elements of the normal ovarian cortex. These elements were, however, ultimately derived from the germinal epithelium of the developing ovary. The ultimate origin of the post-irradiation tissues of the animals X-rayed before and after the attainment of maturity was therefore the same. In the former they were derived directly from the germinal epithelium, in the latter from the epithelial elements composing the cortex. It would appear that in this fact lies the explanation of the similarity in structure and function of the tissues in the three series of animals.

(*b*) *The Anovular Follicles.*

The cords of the second proliferation described in the M and BX series are undoubtedly identical with the anovular follicles described in this paper. In the M and BX series they were derived directly from the germinal epithelium long after irradiation, when the follicles had disappeared, and they never contained an oocyte. In the AX animals they were formed, immediately after irradiation, from small follicles by the degeneration of the oocyte and the growth of the membrana granulosa cells. The term anovular follicles, previously avoided, is therefore justified in the present paper.

The resemblance of these anovular follicles to the "spermatic tubules" described by Hammond in the inbred female rabbit, and to the "spermatic tubules" of certain free-martin gonads was discussed previously. It was concluded that the structures in the irradiated mouse ovaries were the same as those in Hammond's inbred rabbits, and were possibly similar to the structures figured by various authors in the free-martin. They were also compared to the sterile tubules of undescended testes. It would appear therefore that follicles without germ-cells bear a striking resemblance in many respects to spermatic tubules without germ-cells. We are inclined to think now that such structures should be considered as changed towards an intermediate type, and not as indicative of a tendency towards sex-reversal, as was suggested previously by Hammond (3) and accepted by us.

The AX series furnish no evidence concerning whether or not these anovular follicles take part in the regulation of the œstrous cycle as they are present in all the cases. They were shown to be inactive in this respect in the M mice and BX mice (1, 2), and there is no reason for supposing otherwise in the present case.

(c) *The Regulation of the Œstrous Cycle.*

The present series of experiments was carried out primarily with a view to determining whether or not a hiatus occurred in the œstrous cycle after irradiation. In view of the changes in the immature ovary after irradiation, it was thought that such a hiatus, marking the period between the end of the functional activity of the old ovarian tissue and the beginning of that of the post-irradiation proliferation, might occur. To detect such a phenomenon it was necessary to use adult animals in which the cycle was present at the time of irradiation. The œstrous histories of the present series of animals, described elsewhere by one of us (6), demonstrate that no such temporary cessation of the cycle occurred. This result is in keeping with the unexpected absence of proliferation from the germinal epithelium after irradiation, and would appear to indicate that the tissues which produced the œstrin in the normal ovary continued, without intermission, to do so in the sterilised organ. The significance of this conclusion is of fundamental importance, because it indicates, from another aspect, that the maturing follicles do not provide the rhythmic mechanism by which œstrus is produced. The œstrous rhythm is maintained by the irradiated ovary during the time when the follicles are degenerating, and after they have completely disappeared as organised structures. In view of the fact that it was impossible to distinguish between the follicular and interfollicular elements in the sterilised ovaries the production of œstrin cannot be ascribed to one only of these. Either, perhaps both, must produce it, as their derivatives, together with the corpora lutea, constitute, with the exception of the vessels and connective tissue trabeculæ, the entire sterilised ovary. The inactivity, in this respect, of the persistent corpora lutea has been shown previously, and will be dealt with in a later paper in relation to lactation. Œstrin production in the normal mouse can be ascribed, therefore, to the epithelial elements of the cortex, but the exact location of these in either the follicular or interfollicular tissue, or in both, is not indicated by the results recorded in this paper.

V.—SUMMARY.

1. Seventy-five per cent. of the animals killed upwards of five weeks after irradiation when adult were sterilised.

2. No hiatus occurred in the œstrous cycles following irradiation.

3. No post-irradiation proliferations from the germinal epithelium, like those described (1, 2) in the animals X-rayed before puberty, were observed.

4. The elements already forming the cortex at the time of irradiation persisted and constituted the tissues of the sterilised ovary. These were shown to be the elements of the follicles and interfollicular tissue originally derived from the embryonic germinal epithelium.

5. It was concluded that these elements produced œstrin in the normal ovary and continued to do so without interruption after irradiation.

6. "Anovular" follicles were formed from the smaller follicles, by the degeneration of the oocytes and the growth of the membrana granulosa cells, immediately after irradiation. These were identical in every respect with the cords of the second proliferation which were formed in the animals X-rayed before puberty. The latter, however, were formed after the follicles had disappeared, directly from the germinal epithelium, and never contained an oocyte.

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DESCRIPTION OF PLATES 15 AND 16.

GUIDE LETTERS.—*A.F.*, Anovular follicle. *B.V.*, Blood vessel. *C.L.*, Corpus luteum. *D.F.*, Degenerating follicle. *G.E.*, Germinal epithelium. *I.F.*, Interfollicular tissue. *M.G.*, Membrana granulosa. *N.F.*, Normal follicle. *O.*, Oocyte. *T.A.*, Tunica albuginea. *T.E.*, Theca externa. *T.I.*, Theca interna.

PLATE 15.

FIG. 1.—AX 13 showing absence of small oocytes and corpora lutea. Many follicles degenerating and interfollicular tissue increasing. Three days after irradiation. × 36.

FIG. 2.—AX 47 showing enormous increase of interfollicular tissue and absence of follicles and corpora lutea. 55 days after irradiation. × 36.

Fig. 3.—AX 30 showing corpora lutea and interfollicular tissue but no follicles. This animal copulated six days after irradiation and produced and reared a litter. 67 days after irradiation. × 36.

Fig. 4.—AX 34 showing vacuolated interfollicular tissue. 67 days after irradiation. × 36.

Fig. 5.—AX 38 showing increase in interfollicular tissue beginning and absence of small oocytes. Two days after irradiation. × 36.

Plate 16.

Fig. 1.—AX 38 showing two small anovular follicles and one large follicle, containing a degenerating oocyte, in which the theca interna and membrana granulosa elements are tending to merge with the surrounding interfollicular tissue. Two days after irradiation. × 380.

Fig. 2.—AX 7 showing anovular follicles and hypertrophied interfollicular tissue. 73 days after irradiation. × 238.

Fig. 3.—AX 47 showing thin inactive germinal epithelium and continuous tunica albuginea. Large-celled luteal-like interfollicular tissue. 55 days after irradiation. × 370.

We are indebted to Mr. F. J Pittock for the photomicrographs reproduced in these plates.

Ovarian Regeneration in the Mouse after Complete Double Ovariotomy.

By A. S. Parkes (Beit Memorial Research Fellow), Una Fielding (Demonstrator of Anatomy), and F. W. Rogers Brambell (Fellow of the International Education Board).

(Communicated by Prof. J. P. Hill, F.R.S.—Received February 9, 1927.)

(From the Department of Physiology and Biochemistry, and the Department of Anatomy University College, London.)

[Plates 17 and 18.]

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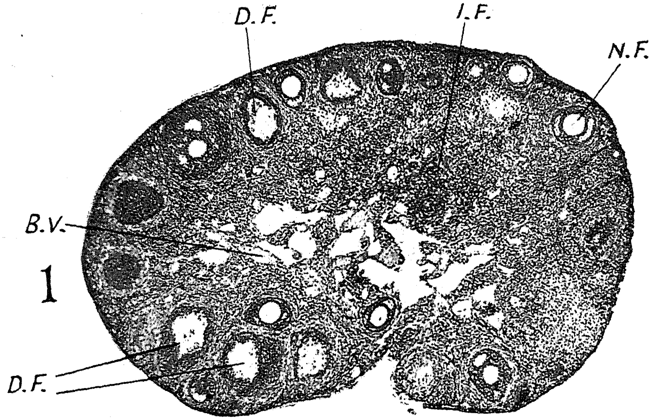
I. Introduction.

The fact that after unilateral ovariotomy the remaining ovary hypertrophies and undertakes the work of two ovaries has been recognised for many years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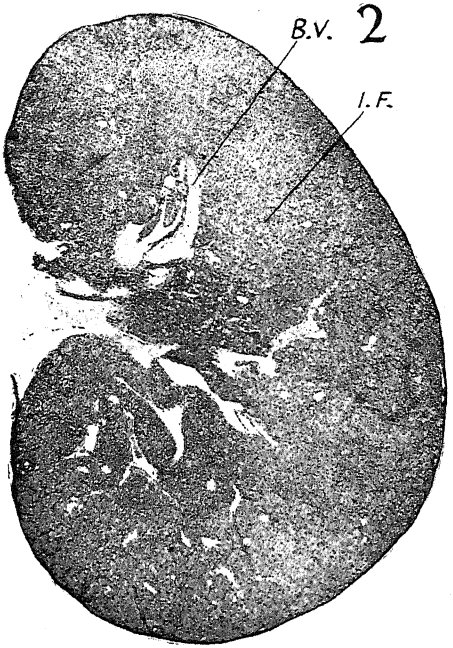
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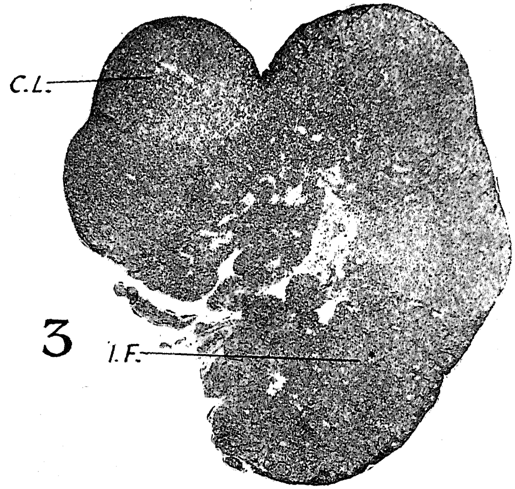
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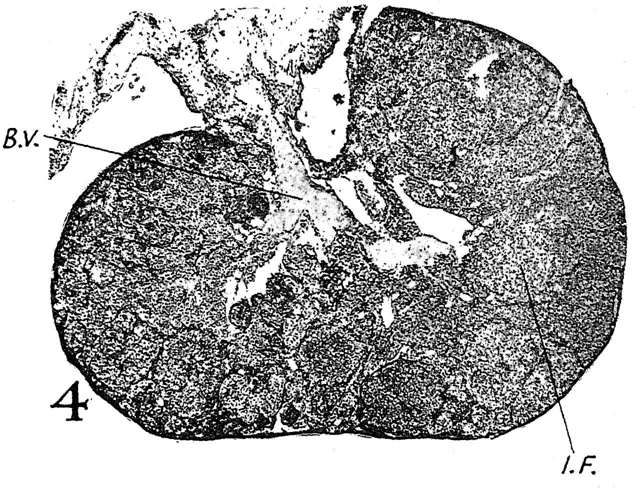
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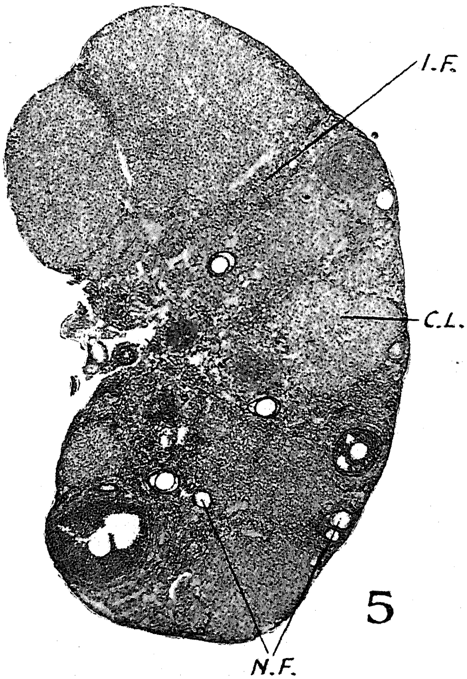
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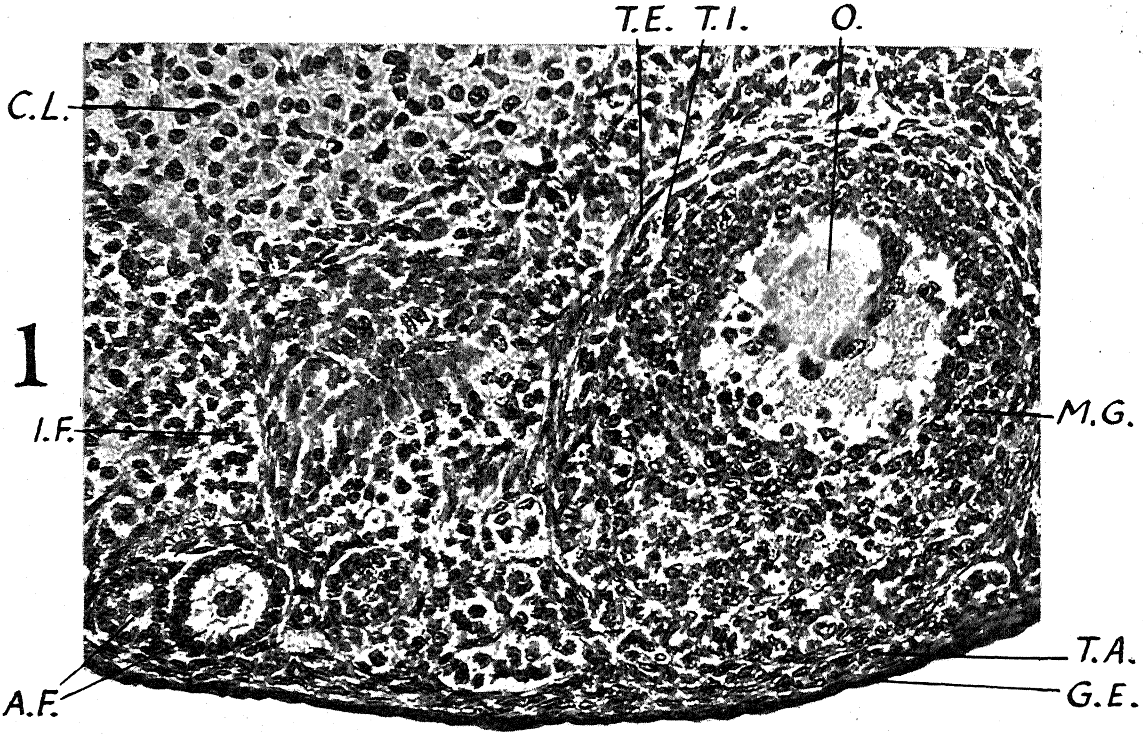
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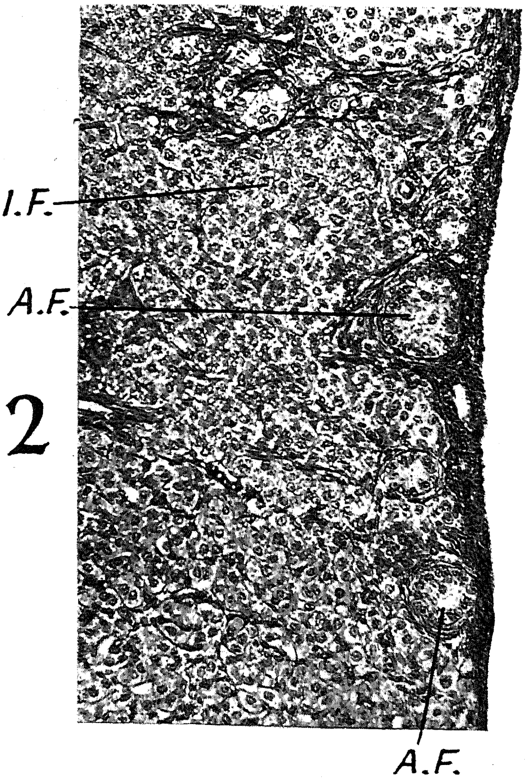
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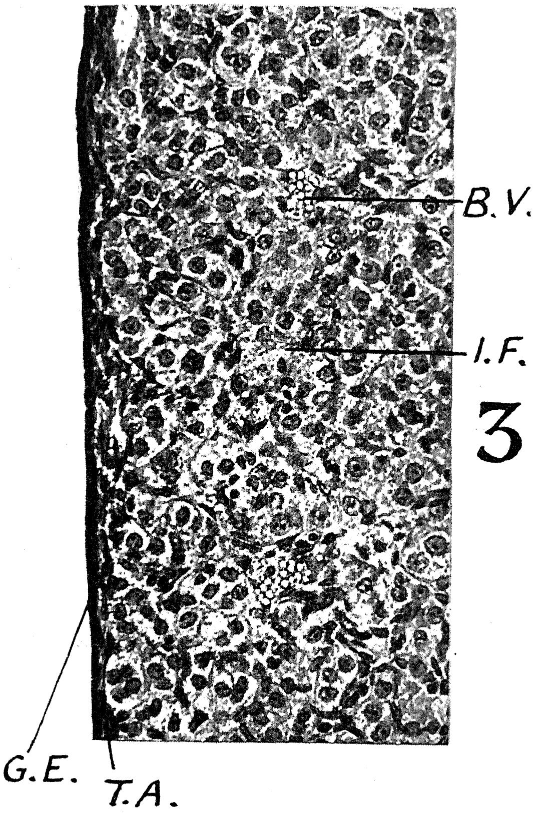
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(Carmichael and Marshall (6), Arai (2), Lipschütz and Adamberg (14)), and in the same way it has been found that ovarian fragments are capable of undergoing intensive hypertrophy to produce appreciable amounts of ovarian tissue (Asdell (3), Hartmann (12), Lipschütz (13)). The regeneration of ovarian tissue after complete double ovariotomy, however, has until recently rarely been considered as a serious possibility, the evidence that such re-formation can take place having been scanty and usually explained on the grounds of incomplete removal of the ovaries.

In 1911 Castle and Phillips (7) reported 10 cases of ovarian regeneration after ovariotomy among 74 guinea-pigs and 17 rabbits, and in three of these young were produced. Subsequently, the authors (5) state that of a total of 141 ovariotomised guinea-pigs, 11, of which three produced young, showed regeneration of ovarian tissue. They also note that in cases of ovarian regeneration the usual ovariotomy effects are not found.

Later, Davenport (9) reported the results of an extensive series of ovariotomies on mice, most of them, however, being only unilateral operations. Where the entire ovary, capsule and Fallopian tube were removed the results showed regeneration (as detected by *post-mortem* macroscopic examination) in four out of 23 cases* of unilateral ovariotomy, and in one (both sides) out of six of double ovariotomy. Where the ovary only was removed, capsule and tube being left as intact as possible, regeneration was found in 37 out of 55 cases of unilateral ovariotomy and in 15 out of 18 of complete ovariotomy, three of the 15 showing regeneration on both sides. Considering the results as percentages, therefore, the following regenerations were obtained :—

	Per cent.
Ovary only removed—	
Unilateral	67·2
Complete	83·3
Ovary, capsule and tube removed—	
Unilateral	17·0
Complete	16·8

Davenport summaries his results as follows : "Regeneration of the ovary in mice is less apt to occur when capsule, tube and part of the fat body are removed than when the ovary alone is removed by cutting its stalk and picking it out of the capsule with least damage to the latter. Regeneration occurs equally in mice that were operated upon when only a month old and in those which were three or four months old. Regeneration may take place within a month or two after operation, but apparently the proportion of regenerated mice increases until six months have passed. Adhesions and minor infections do not inhibit the re-formation of the ovary, and such re-formation is neither facilitated nor inhibited by pregnancy [in case of unilateral ovariotomy]."

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* This citation of results omits four cases considered by Davenport to be unreliable and also operations where the ovaries were classified as "mutilated" or partially or "doubtfully" removed.

No histological examination was attempted by Davenport.

Quite recently Frank and Goldberger (11) have referred to the occurrence of ovarian regeneration, but no details are given.

The observations embodied in the present paper were made as the result of ovariotomising large numbers of mice for the testing of ovarian extracts. Owing to apparent regenerations in certain of the first batch of mice used for this purpose it was decided to make a histological and physiological study of the phenomenon, the importance of which, both from a genetical standpoint and as a complicating factor in the testing of ovarian extracts, seemed to warrant a somewhat detailed investigation.

We are much indebted, as previously, to Prof. J. P. Hill, F.R.S., and to Dr. F. H. A. Marshall, F.R.S., for their active interest in the work. The mice were from the colony maintained with the aid of a grant to A. S. P. from the Government Grants Committee of the Royal Society, and the histological expenses were defrayed from another grant from the same source to F. W. R. B.

II. Material and Technique.

The material available consists of 121 double ovariotomised mice. The operation of ovariotomy was performed by making an incision through the skin and body wall above the ovarian region on each side of the back. The Fallopian tube was then ligatured, together with the ovarian artery and vein, and the major part of the fat body as well as the capsule and ovary were excised. Ordinary surgical silk was originally used for the ligaturing, but where it was desired subsequently to examine the ovarian region for regeneration, the presence of these ligatures was found to be an inconvenience in sectioning. Human hair was finally found to be much better on this account and was also found to cause fewer stitch abscesses. There can be little doubt about the completeness of the operation as performed, but in all the later cases the excised ovaries were fixed in order that the completeness of the removal could, if necessary, be subsequently checked by serial sections.

Detection of Regeneration.

The work recorded in the past appears to have been based upon two criteria of regeneration: (*a*) macroscopic appearance of regenerated tissue, and (*b*) the occurrence of pregnancy after double ovariotomy. Both of these criteria are clearly unsatisfactory. The ovariotomised mouse usually becomes very fat, especially in the region of the ovary and kidney. This accumulation of fat, together with the possible presence of stitch abscesses and the diffuse nature of regenerated ovarian tissue, makes accurate detection by macroscopic examination almost impossible. In the second place, pregnancy after double ovariotomy, though a clear indication that regeneration has taken place, cannot be considered to be a regular symptom. The damage done to the Fallopian tube during the removal of the ovary, especially in a small animal such as the mouse, must in any case often be sufficient to prevent effectively the passage of ova to the uterus. The operation as described above entirely precludes the possibility of such passage. In many cases, also, the regenerated tissue appears to be almost entirely surrounded by fat body, which must prevent the normal dehiscence of the ova. The capacity to become pregnant must therefore be comparatively rare among ovariotomised animals with regenerated ovarian tissue, and thus a large percentage of regenerations would remain undetected if the capacity to become pregnant were used as the sole symptom.

The ultimate criterion of regeneration is, of course, the histological detection of ovarian tissue after complete removal of both ovaries, and a possible method would, therefore, be to section the entire ovarian region of every ovariotomised animal after *post-mortem* examination. This method, however, has two disadvantages. In the first place, the labour of sectioning a large amount of tissue from every animal would be very considerable, and, secondly, it is of great value in experimental work to be able to detect regeneration without killing the animal. Most of the ovariotomised animals were used periodically for the testing of ovarian extracts, and daily vaginal smear examinations were made in all cases for the detection of œstrus (Allen (1)). As a result of these examinations it was found that non-injected ovariotomised mice occasionally showed signs of œstrous symptoms. This fact, taken in conjunction with the observation that the cycle normally stops completely after ovariotomy, and with the supposition that regenerated ovarian tissue would perform the normal ovarian endocrine functions, led us to believe that the spontaneous appearance of œstrous symptoms in the ovariotomised animal might be used as the initial indication of ovarian regeneration. Since early experience showed the reliability of this criterion, we have in all cases used the return of the œstrous cycle after ovariotomy as the first evidence of ovarian regeneration. By this method of detecting its occurrence in the live animal we have been able in certain cases to sterilise the regenerate ovary by X-irradiation. Diagnosis on the intact animal, however, was always checked by histological examination.

In non-injected mice the detection of spontaneous œstrous symptoms presented no difficulty, but in the mice used for testing ovarian extracts, detection was less simple. Quite apart from the question of studying the phenomena of regeneration, however, detection of spontaneous œstrus was imperative from the point of view of accuracy in the technique of testing. Tests made on animals found to have regenerated ovarian tissue were considered to be of no value. In injected animals two phenomena were considered to be suggestive of regeneration. The reaction of the ovariotomised mouse to injection of œstrin is quite definite. An animal injected will show pro-œstrous symptoms in 36 to 48 hours, *i.e.*, on the following morning but one. At the next daily examination pure cornified material is obtained, and the duration of cornification is directly proportionate to the number of mouse units given. It occasionally happened, however, that an injected mouse came into œstrus at a time incompatible with it being the result of injection. This was considered as a clear suggestion of regeneration, and in such cases injections were stopped and the cycle examinations continued. Further occurrence of œstrus was considered to be definite, and led to the animal being killed or further examined in order to study the cycle after regeneration. Secondly, in other cases an injection produced a degree of cornification quite out of keeping with what was already known of the activity of the extract, and further injection into such animals was stopped.

Finally, the occurrence of copulation in ovariotomised mice, even in those receiving injections of ovarian extracts, is of considerable interest. Most of the ovariotomised test animals were not kept with males, but, nevertheless, 92 periods of œstrus, as diagnosed by the vaginal smear technique, have been induced in mated animals by injections of ovarian extract. Of these periods of œstrus only 7, or less than 7·6 per cent., have been accompanied by copulation, and of these three occurred in animals found to have regenerated ovarian tissue.

These results clearly indicate that copulation is not a usual reaction to œstrin injection, and, in addition, it was originally thought that copulation might be used as an index of regeneration. Three animals, however, copulated at induced œstrous periods without giving any other suggestion of regeneration. No spontaneous œstrous symptoms appeared and no histological evidence of regeneration could be found. In view of these results it would appear that copulation does occur, though rarely, after œstrin injection in the non-regenerate ovariotomised animal, and its occurrence, though suggestive, is no absolute criterion of regeneration. Copulation, of course, takes place freely at the spontaneous œstrous periods experienced by mice with regenerate tissue, and copulation after injection in such animals may be considered to be analogous with the œstrus and copulation which occur during diœstrus in the normal intact mouse after injection of œstrin.

Using the return of the œstrous cycle as the chief symptom, it has been possible to detect regeneration in the live animal with comparative ease, but in all suspected cases the ultimate criterion of microscopic examination, after *post-mortem* examination, has been applied.

The results of the histological examinations show that, excluding OVS 14, in only two cases was suspicious œstrus behaviour unconfirmed by histological examination, and in view of the diffuse nature of regenerated tissue, it is probable that it was present but escaped removal in these cases.

Histological Technique.

The ovaries removed operatively were not preserved at first. After some cases of regeneration had occurred it was realised that, had they been preserved, the ovaries removed would have provided indisputable proof of the thoroughness of ovarian extirpation. The ovaries removed at later operations were fixed immediately in Bouin's fluid and stored in 90 per cent. alcohol in tubes. When a case of apparent regeneration occurred, they were embedded and cut. Sometimes the superfluous fat and the capsule were removed when embedding, and consequently the material finally examined was actually less than what had been excised. Complete serial sections were made of each ovary and were stained with either Ehrlich's hæmatoxylin and eosin or iron-hæmatoxylin. When the animal was killed, the tissues surrounding the regions the ovaries had occupied were dissected out. These consisted of a portion of the body wall and fat body, together with the remains of the Fallopian tube and any adhesions involved in the area. The superfluous fat was pared away to some extent to lessen the labour of cutting. The uteri were also preserved. The material was fixed in Bouin's fluid and complete serial sections were made of each ovarian region and stained with Ehrlich's hæmatoxylin and eosin, or iron-hæmatoxylin, or by Pasini's method.

The measurements of the regenerate tissue recorded were made from the slides.

III. Description of Cases.

The histories of the animals showing regeneration are dealt with below :—

OVS 8.—This animal was examined for 38 days after ovariotomy. Œstrus was observed at the time when the operation was performed, and cornification was observed on the two following days. This observation, which has been confirmed on other animals, is interesting in that it shows that the stimulus to cornification is applied some time anterior to the actual event. Four injections of ovarian extracts were made into this animal. The first three were followed by perfectly normal reactions, and none were attended by copulation. The fourth, however, was followed by cornification of the vaginal epithelium within a few hours and by copulation three days later. Since this reaction was most suspicious, autopsy was performed. Each ovarian region was so covered with fat that macroscopic examination was of little use. Microscopic examination, however, revealed the undoubted existence of ovarian tissue. The ovaries of this animal were not preserved.

The regenerate tissue removed at autopsy (Plate 17, fig. 3) measured 1·58 × 1·26 × 0·66 mm. It was oval in section with a regular, clearly defined and rounded contour, and was situated close to the cystic extremity of the Fallopian tube on one side. The mass was completely surrounded on all sides by the tissues of the tube and the fat body. The vascular supply was ample and well established. A periovarian cavity separated a considerable area of the surface of the ovarian tissue, on the side distal to the tube, from the fat body. This cavity was lined by a single layer of cubical epithelium. The epithelium which covered the free surface of the ovarian tissue and that which covered the opposite surface of the fat body were fused around the margins of the periovarian cavity (Plate 18, fig. 2) to form a solid epithelial band which extended round the regenerate tissue. Mitotic figures were not found in this epithelial band, but a number of epithelial cells in the ovarian tissue immediately beneath it in places suggested that it might have been proliferating. A layer of fibroblasts three to five cells thick extended around the regenerate tissue immediately within this layer and constituted a well-marked tunica albuginea.

There were about 30 follicles, each containing an oocyte, in the ovarian tissue. One of these contained an apparently normal oocyte and was almost mature in size, but was compressed and flattened by the pressure of the adjoining corpora lutea atretica. The others varied in size from primordial follicles, consisting of a single layer of flattened epithelial cells around a small oocyte, to follicles, three cells thick, with an antrum beginning to form and enclosing a full-grown oocyte. The nuclei of all the oocytes were in the dictyate stage. Their origin could not be determined as none were observed in process of formation.

Two corpora lutea atretica were present which, with the one large follicle, constituted the major part of the regenerate tissue. They were old and retrogressing. In the centre of each was a cavity, containing the remnants of the zona pellucida, of the oocyte, and of some of the cells of the discus proligerus, surrounded by an old blood clot.

The small amount of stroma present between the follicles and corpora lutea atretica consisted, as in the normal ovary, of epithelial cells, connective-tissue elements and fibroblasts. Polymorph leucocytes were fairly common in the regenerate tissue and, close by, in the immediate vicinity of the ligature. Pigment was present in small quantities in the cells around the periphery of the regenerate tissue. A group of cords, resembling rete, were situated close to, and were continuous with, the ovarian tissue at a place where the tunica albuginea was not formed (Plate 18, fig. 3).

OVS 9.—This animal was observed for 40 days, during which time three injections of ovarian extract and one injection of testis emulsion were given. The first induced œstrous period was followed by copulation 13 days after ovariotomy. Otherwise the symptoms of induced œstrus were normal in all three cases. Thirty-nine days after ovariotomy, however, and 17 days after the last injection of ovarian extract, the animal came into spontaneous pro-œstrus and the next day into œstrus, and was then killed. The ovaries of this animal were not preserved.

The regenerate ovary removed at autopsy (Plate 17, fig. 1) measured 2·0 × 1·71 × 1·38 mm. It was rounded and regular in contour and was situated on one side in a bend of the cystic tube. It was completely surrounded on all sides by the tissues of the tube and the fat body. The regenerate ovary was attached in one place to the tissues of the tube by a definite hilum. The hilum, which was similar to that of a normal ovary, was composed of loose fibrous connective tissue, containing numerous blood vessels and lymph lacunæ, and a few cords or tubules like rete.

The blood vessels which entered at the hilum furnished an ample vascular supply. There was no periovarian cavity developed.

The fat body was separated from the ovarian tissue by a thick fibrous sheath, which stained intensely blue by Pasini's method and contained many fibroblasts. A peripheral zone of cells, probably comparable to the germinal epithelium of the normal ovary, was in contact with the inner surface of the sheath.

It varied from one to several cells thick, and, in places, was entirely absent. The component cells of this layer were large and glandular in appearance and resembled normal theca-interna elements. Within this was a tunica albuginea of fibroblasts a few cells thick. This layer was difficult to distinguish from the sheath where they came in contact, although it was not so fibrous nor did it stain so deeply. These three layers were absent in the hilar region and were poorly developed in one place where the fat body was almost in contact with the ovarian tissue. The surface of one corpus luteum appeared to be at one point in direct contact with the outer sheath, and, in another place, near the hilum, the tube was only separated from the ovarian tissue by the " epithelial " layer and the tunica albuginea.

The ovary contained a number of follicles enclosing oocytes. Three of these were nearly mature. The others varied from primordial follicles to those with a small antrum ready to enter on the final growth stage. One small follicle was buried in the fibrous outer sheath, the inner layers of which separated it from the main ovarian mass. The nuclei of all the oocytes were in the dictyate stage and there was no evidence of their origin.

Six corpora lutea were present. All contained a central blood clot, and in some the remnants of the zona pellucida could still be detected. It is probable, therefore, that they were all corpora lutea atretica. Extravasated blood, as well as the central blood clot, was present in one of them. The six corpora lutea atretica were in three different stages, retrogression being far advanced in the oldest.

Cells similar to those composing the peripheral " epithelial " band occupied the areas between the follicles and corpora lutea. Pigment occurred in the cells of the outer sheath, the periphery of the ovarian tissue and the hilum. Leucocytes were practically absent from the ovarian tissues.

OVS 13.—This female was observed for 22 days only. During this time three injections of ovarian extract were made. The first two were followed by perfectly normal reactions, but 18 days after ovariotomy and the same day as the third injection was made cornification appeared and lasted for four days. On the day before killing the vaginal smear had a typical diœstrous nature. The ovaries were not preserved.

The ovarian tissue removed at autopsy (Plate 17, fig. 2) measured 0·68 × 0·76 × 0·69 mm. It was subspherical in shape and situated on one side close to the old ligature and the cystic remains of the Fallopian tube. It was completely surrounded on all sides by the tissues of the tube and the fat body. The vascular supply appeared good.

A fibrous sheath, varying in thickness from 60 to 100 μ, surrounded the ovarian mass. Small islets of "epithelial" cells were interspersed in places between the fibroblasts of this layer. These were more plentiful on the side next the ovarian tissue, where they merged to form a continuous peripheral zone. This peripheral zone was almost absent in some places and about 90 μ thick in others. The "epithelial" cells were the chief components, but connective-tissue elements, arranged in a circular direction, were also numerous. Cells in mitosis were not observed. Capillaries were present in this layer, and, in one place, several large fat-laden adipose cells similar to those in the surrounding fat body. In another place where the band was well developed (Plate 18, fig. 1), small fissures or clefts occurred in it. The cells were arranged around each of these to form a single-layered cubical epithelium. These may represent the early stages in the formation of a periovarian cavity such as that described in OVS 8.

Nine small oocytes in primordial follicles occurred in this peripheral epithelial band. The nuclei of all the oocytes were in the dictyate stage, and there was no evidence of their origin. One large corpus luteum atreticum, about 0·65 mm. in diameter, occupied the centre of the ovarian mass and, with the peripheral epithelial band, composed the whole of it. In the centre of the corpus a blood clot surrounded a cavity which contained the crumpled zona pellucida of the old oocyte. This corpus luteum atreticum was beginning to retrogress. Leucocytes were scarce in the ovarian tissue, though plentiful in the neighbourhood. A small amount of pigment occurred in and near the periphery of the regenerate tissue.

OVS 14.—This animal was examined for 21 days after ovariotomy. One injection of ovarian extract, followed by normal œstrous symptoms, was given. Thirteen days after ovariotomy spontaneous œstrus appeared, and this was repeated six days later, cornification lasting at the last œstrous period until the time of killing. The ovaries were not preserved. Owing to an accident while washing, the ovarian regions, which had been removed for histological examination, were unfortunately lost, but this case must be considered as a typical case of regeneration.

OVS 52.—This animal was observed for only 22 days. Copulation took place nine days after ovariotomy, following injection of ovarian extract, and this was repeated 13 days later, after two days of spontaneous œstrus, which appeared to be clear evidence of the presence of ovarian tissue. The animal was then killed, and microscopic examination revealed the undoubted presence of ovarian tissue.

Owing to the speed with which regeneration took place, this case appeared superficially to be one suggestive of incomplete removal of the ovaries. This possibility, however, was effectively disposed of by examination of complete serial sections of the original ovaries. On examination, the ovaries were found to be intact, with a considerable portion of the mesovarium attached to the hilum of each.

The regenerate ovarian tissue (Plate 17, fig. 4) measured 0·924 × 1·05 × 0·918 mm. It was situated on one side close to the cystic remains of the tube and the old ligature. A tag of tissue, which resembled rete cords, attached it to the wall of the tube. Except for this slender attachment it was completely surrounded on all sides by the fat body. The vascular supply appeared scanty, which may explain the unhealthy appearance of the tissues.

A fibrous layer separated the ovarian tissue, which it surrounded, from the fat body. No periovarian cavity was present and no definable peripheral "epithelial" band.

One large follicle, 750 μ in diameter, with a degenerate oocyte, occupied a large part of the ovarian mass. A number of other follicles, varying from primordial follicles to ones with a very small antrum, were present and contained normal oocytes. The nuclei of all the oocytes were in the dictyate stage. No corpora lutea were present.

The tissue between the follicles was composed of large vacuolated glandular-looking cells, which did not everywhere appear healthy. In places they were arranged in groups or cords. The theca interna of the large follicle was united by a strand with the inter-follicular tissue, which it closely resembled. This vacuolated tissue was very like that which composed the ovaries of X-rayed mice (4, 5). Mitotic figures were not observable in the ovary. Leucocytes and pigment were practically absent from the regenerate tissue, although the former were plentiful in the neighbourhood. A few small lenticular islands of "epithelial" cells were present in the connective tissue surrounding the mass.

OVS 75.—This animal was observed for 178 days. Ovariotomy was followed by 24 days of complete absence of œstrous symptoms, which shows fairly conclusively that complete removal of functional ovarian tissue had been effected. During the next 17 days four injections of ovarian extracts were made, and these were accompanied by the normal reactions. A fifth injection was then made, and this was followed by intensive cornification on the forty-sixth day after ovariotomy. This period of cornification lasted for 15 days, and was clearly not due to the injection. No further testing, therefore, was done on this animal. The subsequent history was one of periodic spells of intense cornification, and may be summed up as follows (figures for the length in days of cornified periods being given in italics) :—

(4 + *10*) + (5 + *20*) + (5 + *2*) + (2 + *5*) + (3 + *7*) + (3 + *8*).

At this stage the animal was mated, and in the following cycle charts the letter P shows the presence of a vaginal plug indicating copulation :—

(2 + P + *2*) + (1 + P) + (4 + *9* + P + *4*) + (2 + *3*) + (2 + *14*).

Two main points of interest arise from this œstrous history—

(*a*) the remarkably long periods of cornification, and

(*b*) the occurrence of copulation in the middle of a cornified period, and in the middle of what had every appearance of being a diœstrous period.

If, as we have every reason to believe, the alternate presence and absence of cornified matter in the vagina is the expression of an ovarian rhythm, these facts seem to indicate a considerable degree of derangement in the ovarian cycle, and also suggest that regenerate ovarian tissue may be erratic in its cyclic activity. This problem will be more fully discussed in the next section in dealing with the correlation between the ovarian and vaginal cycles in these regenerate animals.

The ovaries were preserved and complete serial sections of them were made. On examination, the ovaries and a large portion of the mesovarium, attached to each hilum, were found to have been removed intact, except for a small tangential slice off the surface of two adjoining corpora lutea in one ovary. The cut surface was covered by a blood clot, which indicated that it had been lost during ovariotomy or immediately after the ovary had been removed from the animal and before it had been placed in the fixative. It was not known whether the imperfect ovary came from the side on which the ovarian tissue was regenerated.

The regenerated ovary (Plate 17, fig. 5) measured 1·88 × 1·55 × 1·32 mm. It was in close relation to the end of the Fallopian tube and formed a cap over it. The concave side which embraced the end of the tube formed a definite hilum, which consisted of loose connective tissue and enclosed blood vessels, lymph lacunæ and rete-like tubules. The ovarian tissue was embedded in the fat body, from the surface of which it projected slightly on the side away from the hilum. The vascular supply was good.

No periovarian cavity was present. A fibrous sheath was developed in places between the fat body and the ovarian tissue, and was especially well formed over the projecting surface of the latter. Elsewhere the ovarian tissue was in direct contact with the fat body. No definite peripheral "epithelial" band was present.

No follicles or oocytes were present in the mass. One large corpus luteum, which contained a central blood clot and areas of extravasated blood, was present. It was clearly retrogressive, being infiltrated with leucocytes and showing signs of fibrosis. Another small luteal vestige was present, very degenerate and fibrosed.

The stromal tissue was composed of large vacuolated, glandular-looking cells interspersed with fibroblasts. It was very like the tissue of some X-rayed ovaries (4, 5). The peripheral and hilar regions were more fibrosed than the central and contained pigment. Leucocytes were plentiful in the stroma as well as in the luteal tissue.

OVS 82.—This animal was examined for 147 days after double ovariotomy. During the first 111 days a number of ovarian extracts were injected, normal results following in each case except the last. On this occasion the reaction seemed to be very marked when considered in relation to what was already known of the extract. The extract in the same amount was therefore tested on another animal and found to be inactive. Regeneration was therefore assumed to have taken place in OVS 82, and further observation without injection was made. Two more periods of cornification were observed, the animal being killed when the last had continued for 22 days. The spontaneous cornification history of this animal is therefore as follows :—

$$\mathit{3} + 4 + \mathit{2} + 4 + \mathit{22}.$$

The ovaries removed at operation had been preserved and complete serial sections of them were made. On examination, both, together with the portion of the mesovarium attached to the hilum, were found to be intact and were almost entirely surrounded by tissues of the fat body.

The regenerate tissue measured 0·95 × 1·32 × 0·844 mm. It was situated on one side in the fat body close to the remains of the tube, to which it was attached by a small hilum. This was composed, as in the normal animal, of loose connective tissue containing blood vessels, lymph lacunæ and several small tubules suggestive of rete. The regenerate mass was surrounded by the tissues of the fat body.

No periovarian cavity was developed, nor was there a well-marked fibrous sheath around the ovarian tissue. The outer layers of the latter were composed of a few large glandular-looking cells, like those in the other cases, and fibroblasts.

Two follicles were present in the tissue. One was about the size of a normal mature follicle, but the antrum was filled with blood. The other was slightly larger, with the degenerate oocyte free in the antrum. Both were obviously in the process of forming cysts.

One corpus luteum atreticum with a central blood clot surrounding a cavity, which contained the remains of the zona pellucida of the oocytes, was present.

There was little or no stromal tissue between the follicles and corpus luteum atreticum.

OVS 54 was observed for 31 days. Injections of ovarian extract were made in the first fortnight, two of which produced œstrous symptoms. The reaction to the last injection appeared to be more intense than could be accounted for by what was already known of the activity of the extract, and the animal was therefore kept under examination without further injection. Nine days after the end of the previous period of cornification, and 27 days after ovariotomy, spontaneous cornification appeared. After marked cornification for five days, autopsy was performed.

The ovaries were preserved. One has a small tear in the hilum, but no tissue appears to be missing. The other is intact. No regenerate tissue was found in the material removed from the ovarian region at autopsy.

OVS 117.—Observations on this animal were begun 23 days after ovariotomy and were carried on for 44 days. Three injections were given. The first of these produced normal œstrous symptoms, while the second was inactive. The third was followed in four days by cornification, which is longer than the normal reaction time. In addition, the amount of ovarian extract given was shown on other animals to be inactive. The initial diagnosis of regeneration was confirmed by the persistence of this cornification for eight days, when the animal was killed.

The ovaries removed from this animal were both absolutely intact (Plate 17, fig. 6), with the capsules complete around each, and with a large part of the mesovarium and the hilum attached to each. No regenerate tissue was found in the material removed from the ovarian region at autopsy.

OVS 17.—This animal was examined for 112 days after ovariotomy. During the first six weeks of this time three injections of ovarian extract were given, and followed by the normal reactions. On the 49th day spontaneous cornification, lasting for four days, appeared. This appeared to indicate regeneration, and as it seemed possible that the X-ray sterilisation of regenerate tissue might give results of value, the animal, eight days later, was given irradiation amounting to $\frac{1}{8}$ B tint on Levi's pastilles (half the amount required in our experience to produce sterility fairly uniformly). On the 65th day spontaneous œstrous symptoms again appeared, and on the 67th day the exposure to X-rays was repeated. Œstrous symptoms were again experienced on the 74th, 86th and 96th day. At these periods cornification of the vaginal contents was observed to last respectively for one, five and seven days. This animal, therefore, experienced five spontaneous periods of œstrus, the last three being unaffected by the sterility dose of X-rays. The cycle formula runs: 13–9–12–10. The extensive cornification found at the last two periods was the first indication received of the totally abnormal duration of cornification, which was subsequently found to occur occasionally in regenerated animals. The animal was mated during the whole time under examination, but no copulation was observed. The ovaries were not preserved.

The regenerate tissue was situated on one side in the fat body close to the remains of the tube. Its similarity to other X-rayed ovarian tissue was so great that its ovarian character was beyond dispute. Its histological detail will be described elsewhere. In the present paper it is sufficient to state that it was surrounded by a loose fibrous sheath, had a good vascular supply, and was attached to some tubules resembling rete. Oocytes, follicles and corpora lutea were entirely absent.

OVS 23.—This animal had a very similar history to OVS 17, except that no injections were made. Examination was continued for 71 days, and during this time œstrous symptoms were observed on six occasions. On the first three of these cornification was not unduly prolonged, but on the last three it was quite abnormal. The first pro-œstrus began on the 14th day after ovariotomy. Like OVS 17, this animal was also irradiated, the two doses being given on the days following the end of the second and third œstrous periods. The duration of the various parts of each cycle, *i.e.*, diœstrus, pro-œstrus and œstrus, are given below in Table I. For the prolonged "diœstrus" hiatus following ovariotomy, no figure is given, as it cannot be considered to be analogous to true diœstrus.

Table I.

	Diœstrus.	Pro-œstrus.	Œstrus (cornification).	Total.
1st cycle	—	2	4	6+
2nd ,,	4	1	3	8
3rd ,,	4	1	2	7
4th ,,	4	1	8	13
5th ,,	4	1	9	14
6th ,,	2	1	8+	11+

The last period of cornification was apparently incomplete when the animal was killed. From this table it will be seen that the duration of diœstrus and pro-œstrus was fairly regular and normal, whereas in at least three cycles the duration of œstrous cornification was abnormally prolonged. This extensive cornification may possibly be due to the irradiation, but as several non-irradiated mice with regenerated ovarian tissue showed the same type of thing, it hardly seems probable that this is so. OVS 23 was mated with a normal male the whole time during which observations were being made, but no copulation was observed.

The ovaries were preserved. Both have a small tear in the hilum, but no tissue appears to be missing. The regenerate tissue measured 0·588 × 1·17 × 0·894 mm., and was situated on one side in the fat body close to the remains of the tube. There was no periovarian cavity and the tissue was only separated from the fat body by a layer of fibroblasts at most two cells thick. Its similarity to other X-rayed ovarian tissue, as in OVS 17, was beyond dispute. Its histological details will be described elsewhere. No oocytes, follicles or corpora lutea were present, but zones of cells suggestive of persistent follicular elements were found. These surrounded cavities and resembled those described (4, 5) in some X-rayed ovaries.

IV. DISCUSSION.

Evidence of Regeneration.

The proof of the occurrence of ovarian regeneration after complete double ovariotomy obviously involves two distinct demonstrations. First, that the extirpation of ovarian tissue was complete, and, second, that ovarian tissue was present at some time subsequently. The evidence bearing upon the first of these points is dealt with below :—

(*a*) The œstrous cycle in the 11 animals described stopped after double ovariotomy for a longer time than could be accounted for by an unusually long diœstrous period, and since, as shown below, a small amount only of ovarian tissue is required to produce œstrous symptoms, this complete cessation of the cycle suggests complete removal of functional ovarian tissue. This must be qualified by the statement that œstrous symptoms sometimes occur in the mouse up to two days after double ovariotomy. This phenomenon is almost certainly due to the ovaries having exerted the œstrous-producing stimulus before being removed, and the consequent reaction only taking place afterwards. The question will be dealt with more fully in a subsequent paper.

(*b*) The serial sections of the extirpated ovaries show whether or not they were completely removed. This material is only available for six animals: OVS 23, 52, 54, 75, 82 and 117. The value of this point depends on a clear understanding of what is meant by the terms ovary, hilum, mesovarium, capsule, etc. The use of the terms in the present paper can be seen at a glance from the photograph of a cross-section of one of the ovaries (Plate 17, fig. 6) and surrounding tissue removed from OVS 117. In the mouse the hilum is formed by the downgrowth of connective tissue from the Wolffian body through the mesovarium into the ovary. It contains blood vessels, nerves, lymph lacunæ and persistent remnants of mesonephric tubules and rete. Its formation is not contributed to at all by the tissues of the ovary. In five (*i.e.*, OVS 23, 52, 54, 82 and 117) of the six cases available for this point the ovaries and the portions of the mesovarium attached to the hilum were shown to have been removed intact and in one of these cases the capsules also. In the sixth case, OVS 75, a small portion of one ovary containing luteal tissue was absent and may have been left in the animal. There is no evidence, if it was, that it occurred on the side on which regeneration subsequently occurred.

(*c*) Reference should, however, be made to the possibility that third ovaries or accessory ovarian structures, such as have been described by Waldeyer (18) and others, may account for the apparent regeneration. As regards third ovaries, the following remarks may be made :—

(i) The double "ovariotomy" of an animal with a third ovary left behind would presumably not stop the œstrous cycle, as was the effect in all the animals described above.

(ii) The ovarian tissue found after ovariotomy had in no case the relations of the normal ovary.

(iii) Third ovaries and aberrant ovarian tissue can hardly be as common as the occurrence of supposed regeneration after ovariotomy. In the dissection of some thousands of mice we have seen only one case of a third ovary, and in this instance the accessory organ had a common capsule with one of the normal ovaries, and would therefore have been removed at ovariotomy.

It seems improbable, therefore, that the presence of developed third ovaries can account for our results. The second possibility, that small pieces of ovarian tissue, split off from the main body during fœtal life, and undergoing hypertrophy after ovariotomy, might account for the apparent regenerations, is less improbable. The presence of such rudimentary tissue would not prevent the initial cessation of the cycle, and would most likely not be detected in the unoperated mouse on dissection. The histological evidence, however, is against this supposition.

It seems highly probable, therefore, that the occurrence of ovarian tissue after double ovariotomy is not due to the pre-operative presence of third ovaries or of aberrant ovarian tissue.

The evidence demonstrating that ovarian tissue occurred subsequently in these animals is as follows: (*a*) The vaginal changes characteristic of the œstrous cycle recommenced in all the 11 animals, and, in some cases, were accompanied by copulation; (*b*) ovarian tissue was demonstrated histologically in eight of these cases. The material of one (OVS 14) of the remaining three was not available owing to an accident. The sections of the ovarian regions of the remaining two (OVS 54 and 117) failed to reveal any ovarian tissue. It would appear necessary to assume, to account for the recommencement of the œstrous cycle, that it was present in these animals but was missed on dissection.

The evidence under these four headings for all the cases is summarised in tabular form below :—

Table II.

	Proof of removal.		Proof of regeneration.	
	Stoppage of œstrous cycle.	Ovaries removed complete.	Recommencement of œstrous cycle.	Histological.
OVS 8	+	·	+	+
,, 9	+	·	+	+
,, 13	+	·	+	+
,, 14	+	·	+	·
,, 17	+	·	+	+
,, 23	+	+	+	+
,, 52	+	+	+	+
,, 54	+	+	+	—
,, 75	+	—	+	+
,, 82	+	+	+	+
,, 117	+	+	+	—

— Inconclusive negative evidence. · No histological evidence.

Serial sections of both the ovarian regions of four animals, in which the œstrous cycle was observed not to recommence, failed to reveal any regenerate tissue. This evidence, though insufficient, supports the view that the vaginal smear technique affords a sensitive method of detecting ovarian regeneration.

The Structure of the Regenerated Ovary.

Regenerated ovarian tissue was found on one side only, never on both. It was always situated close to the end of the Fallopian tube, usually more or less cystic owing to being ligatured. The ovarian tissue was always completely surrounded by the fat body and the wall of the tube, and in no case was it in contact or even in close proximity to the peritoneal epithelium.

A region resembling the hilum of the normal ovary was present in most cases and joined the ovarian tissue to the connective-tissue sheath of the Fallopian tube. This hilum consisted, as in the normal animal, of loose fibrous connective tissue enclosing lymph lacunæ, the chief vessels supplying the ovarian tissue and some cords or tubules (Plate 18, fig. 3), which resembled persistent rete cords so closely as to leave no doubt of their identity.

A more or less well-formed fibrous sheath was developed in most cases around the ovarian tissue, where the tissue of the fat body came in contact with it.

A peripheral band of cells, resembling the epithelial elements in the early embryonic gonads of the mouse, was, in some cases, differentiated around the ovarian tissue. Sometimes these cells were larger, more vacuolated and glandular-looking and somewhat similar to the theca-interna elements of the mature follicle. In other cases no distinct peripheral band was present. This band appeared, from its nature and position, to represent the germinal epithelium of the normal ovary. It was in contact on the outside with the surrounding fibrous sheath in which small islands of similar tissue occasionally occurred. A tunica albuginea, similar to that of the normal adult mouse ovary, was developed beneath it. In OVS 13 small cleft-like cavities were present in the epithelial band, the cells of which formed a cubical lining epithelium (Plate 18, fig. 1). In OVS 8 a more extensive cleft separated one side of the ovarian mass from the fat body. This cleft was lined on both sides by a cubical epithelium, which was continuous with the solid epithelial band which surrounded the rest of the surface of the ovarian tissue (Plate 18, fig. 2). These two cases appeared to be two stages in the formation of a periovarian cavity and a germinal epithelium from the solid epithelial band.

Oocytes and follicles occurred in all the regenerated ovaries, with the exceptions of OVS 75 and the X-ray sterilised animals OVS 17 and 23. The regenerate ovary of OVS 75 is unique, therefore, in being completely sterile. It is possible, but not probable, on account of the other cases, that this was due to the small portion of luteal tissue, missing from the first ovaries, and possibly left in the animal, being the source of origin. OVS 8, 9 and 52 each contained a considerable number of oocytes and follicles of varying sizes. OVS 13 contained only nine primordial follicles, and OVS 82 two large cystic follicles. All the oocytes had dictyate nuclei, and in no case did any appear to be forming from the epithelial cells or other ovarian elements.

Corpora lutea were present in all the regenerate ovaries with the exception of OVS 52, and the X-ray sterilised animals OVS 17 and 23. They exhibited various stages of retrogression, and in most cases were shown to be formed from atretic follicles by the persistence of the zona pellucida of the oocyte in them. The remaining cases may be assumed to be corpora lutea atretica also, as ovulation must have been almost impossible on account of the surrounding tissues.

The Origin of the Regenerated Ovary.

It remains to consider what tissue gives rise to new ovarian tissue after the old has been completely extirpated. In the absence of very early stages of the regenerative process, this must remain a matter of conjecture. The isolation of the tissue from the peritoneal epithelium would seem to preclude this source. The occurrence of a definite hilar region with rete-like tubules suggests that the stump of the mesovarium, probably containing bits of the rete, was the source. It is, however, as difficult to imagine how the remnants of the mesovarium regenerate ovarian tissue as it is with any other non-ovarian tissue.

Time Relation of Regeneration.

Since the initial diagnosis of regeneration depended on the occurrence of œstrous symptoms, the time relation of regeneration must be considered as the time taken to regenerate enough tissue to produce œstrus. There is evidence, however, that the amount of tissue required need not be large. Many of the regenerate ovarian areas found contained very little non-luteal tissue. In OVS 13, for instance, the regenerate tissue measured 680 $\mu \times 760 \mu \times 690 \mu$, of which the one large corpus luteum atreticum occupied 600 $\mu \times 750 \mu \times 640 \mu$. In this animal pro-œstrus occurred six days before autopsy, so that this corpus luteum apparently represents an attempted ovulation at this time. Since the œstrous-producing stimulus becomes operative at least 36 to 48 hours before œstrus occurs (as will be shown in a later paper), and since at this time the follicle is comparatively small, œstrus must have been produced in OVS 13 by the small band of non-luteal tissue and one follicle. It would seem, therefore, that a comparatively small amount of tissue is capable of elaborating the necessary stimuli for œstrus production. This conclusion, of course, is in agreement with what has been found as the result of sub-total ovariotomy.

In the circumstances the first appearance of spontaneous œstrous symptoms may be considered as a sufficiently accurate guide to the time at which regeneration occurred, the actual beginning of regeneration presumably being in all cases slightly anterior to the first symptoms discernible in the intact animal.

The time (in days after ovariotomy) of the first appearance of œstrous symptoms in the 11 animals in which the cycle recurred, omitting, of course, immediate post-ovariotomy periods, is shown in the following table:—

Table III.—Time of First Appearance of Spontaneous Œstrus after Ovariotomy.

Animal.	Nature of œstrous symptoms.	Time after ovariotomy.
OVS 8	Spontaneous pro-œstrus	34 days.
,, 9	Spontaneous pro-œstrus and œstrus	38 ,,
,, 13	Spontaneous pro-œstrus	17 ,,
,, 14	Spontaneous pro-œstrus and œstrus	12 ,,
,, 17	,, ,,	48 ,,
,, 23	,, ,,	14 ,,
,, 52	Spontaneous œstrus and copulation	20 ,,
,, 54	Spontaneous œstrus	51 ,,
,, 75	Appearance of prolonged cornification	46 ,,
,, 82	Spontaneous œstrus	113 ,,
,, 117	Excessive cornification	37 ,,

This table shows that of the 11 cases, four regenerated within one month of ovariotomy and six more within two months. OVS 82 provides the only case of regeneration after a long period. These results substantiate Davenport's conclusion that regeneration is most common within a month or two of ovariotomy, but that the proportion increases up to six months. It is impossible at the moment to offer any explanation of this variation in the time of regeneration, but it is clear that no critical period exists when regeneration takes place, if at all.

Percentage of Mice with Regenerate Tissue.

Analysis of our material shows that the percentage of mice regenerating ovarian tissue after ovariotomy is considerably less than the percentage found by Davenport, though it is rather above that found by Castle and Phillips for guinea-pigs (7·8 per cent.). In our material, however, it is noticeable that the proportion regenerating is greater in our early cases, and this is probably due to the fact that in the later operations great care was taken to remove as much of the coiled Fallopian tube as possible, in addition to ovary, capsule, hilum, and the top of the tube. The percentage regenerations obtained by Davenport were strictly correlated with the completeness of the removal of these accessory structures. Even so, however, our percentage regeneration (9·1 per cent.) after removal of ovary, capsule, and tube is much less than that shown by Davenport (16·8 per cent.) after the same type of operation. Three possible explanations of this discrepancy may be suggested :—

(*a*) Difference in the technique of the operation may have caused greater damage to occur in our cases.

(*b*) Autopsy was made on many of our ovariotomised animals before a reasonable time for regeneration to take place had elapsed.

(*c*) Failure to detect regeneration by the œstrous cycle technique may have lowered the percentage.

The third of these possibilities seems highly improbable. It is very doubtful whether regeneration would take place without reappearance of the cycle, especially in view of the fact that a number of animals in which no cycle appeared were examined histologically with negative results. In any case the œstrous cycle technique is very unlikely to miss more cases of regeneration than the macroscopic methods used by Davenport. The explanation, if any, of our lower percentage of regenerations must, therefore, be sought in one or other of the first two suggestions mentioned above.

Length of Cycle after Regeneration.

In many cases the œstrous periods and cycles observed after regeneration were perfectly normal phenomena. In other cases, however, notably those showing prolonged cornification, gross irregularities occurred, and it was difficult to sort out the alternating periods of cornified smears and leucocyte smears into anything resembling a normal cycle. This was especially true when, as in OVS 75, copulation took place at apparently meaningless times.

The normal cycle is always considered in our records to run from the beginning of diœstrus (including metaœstrus) to the end of œstrus (including pro-œstrus), and this convention is used in the present paper in considering such cycles as were fairly normal, and in endeavouring to classify the erratic phenomena observed in the cases of animals such as OVS 75. How far such animals can be considered to have a cycle in the ordinary meaning of the word is doubtful, especially as regards the causative mechanism of the cycle; but it is necessary to consider as far as possible the general nature of the cycle after regeneration.

Of animals showing spontaneous œstrus, uncomplicated by concurrent or nearly concurrent injection, at some period after ovariotomy, only five animals had the two or more periods necessary for the observation of one complete cycle, and of these, two, OVS 17 and 23, were irradiated. As, however, it has been shown (16) that a normal cycle persists after irradiation, this fact is of no great importance at the moment. Of the total of five animals, three showed cycles approximating to the normal.

The Cyclic Structures of the Regenerated Ovarian Tissue in relation to the Œstrous History of the Animal.

In considering the relation between the structures found in the regenerate ovarian tissue and the œstrous history of the animals, it is necessary to bear in mind the three following points :—

(*a*) The injection of ovarian extracts may have retarded or facilitated certain ovarian elements. Since, however, animals known to be regenerate were not further injected, this complication does not arise in the case of those kept for any length of time after regeneration was first detected.

(*b*) The enclosure of the regenerated tissue in fat body resulted in the mature follicle failing to ovulate and undergoing atresia with the formation of corpora lutea atretica. This mechanical barrier to ovulation possibly may have had effects on the development of the follicles.

(*c*) The failure histologically to find regenerate tissue in two cases where regeneration was clearly indicated by the œstrous histories seems to indicate that regenerate tissue may rather easily be left in at dissection, possibly owing to it occurring at some distance from the old ovarian region. This consideration has the corollary that even where regenerate tissue was found, it may not all have been removed.

Nevertheless, in the majority of cases it is possible to correlate the recent œstrous history of the animal with the structures found in the regenerate ovary, but in certain instances the supply of follicles and the number of corpora lutea found seem to be inadequate to allow of the supposition that follicular maturation took place at each œstrus observed. In OVS 8 the two corpora lutea found probably date only from the five-day œstrous period, at the end of which the animal was killed. In this case only one spontaneous period was observed, so no further sets of corpora lutea would be expected. In OVS 9, however, the six corpora lutea found were clearly of three different ages. This is difficult to correlate with the observation of only one spontaneous œstrous period, but it may be explained on the grounds that ovulation may have taken place at one of the earlier induced œstrous periods, or possibly on the grounds that a spontaneous period exactly corresponded with an injection. In OVS 13 the corpus luteum found appears to correspond to the spontaneous œstrous symptoms observed six days before autopsy. The complete absence of oocytes, follicles and corpora lutea in the sterilised regenerate mice (OVS 17 and 23) is further evidence that the œstrous cycle is not dependent on the periodic maturation of follicles. In OVS 52, which showed cornification and copulation in the three days previous to killing, the presence of corpora lutea in the regenerate tissue might have been anticipated. This anticipation was not fulfilled, however, but the large follicle with a degenerating oocyte found appeared to be in the initial stages of cyst formation.

In the case of OVS 75 the structures of the regenerate tissue show no sort of correlation with the œstrous history of the animal. The entire absence of follicles and oocytes, and of corpora lutea with one exception, is quite irreconcilable with the previous occurrence of periodic cornification unless it is assumed that maturation of follicles is not essential to œstrus. The occurrence of only one corpus luteum in OVS 82 presents another instance of the failure of the cyclic structures of the regenerate tissue to correspond with the previous history of the animal.

The most peculiar feature of the œstrous phenomena in these animals with regenerate ovarian tissue is the prolonged cornification which is found in some cases, notably OVS 75 and 82. We are informed by Dr. E. A. Doisy that the same thing is occasionally found after ovariotomy in the rat. This prolonged cornification is very reminiscent of the reaction which follows the administration of one large dose of œstrin (Parkes and Bellerby (17)) or a series of small doses. The fact that œstrous symptoms do not last indefinitely in the normal mouse suggests two possibilities : (*a*) That the concentration of œstrin sinks rapidly below the required value once œstrous changes are initiated, or (*b*) that the corpus luteum of ovulation in the mouse exerts an œstrous-inhibiting action. The work on X-ray sterilisation reported elsewhere makes (*b*) highly improbable, so that presumably the former possibility must be considered as a tentative working hypothesis. On this view the prolonged periods of cornification might result from the abnormal retention of a threshold concentration on the part of the œstrous-producing hormone. How this could be brought about is difficult to see, but probably some hyperactivity of the ovarian tissue is responsible. Why, however, regenerate tissue should tend to be hyperactive in the production of œstrin is also obscure.

In a certain number of cases we have observed abnormally prolonged cornification in the untreated mouse, and it is hoped that a systematic examination of such of these cases as occur will throw some light on this peculiar problem.

Does Regeneration occur after Double Ovariotomy in the Human Subject?

Finally some reference may be made to the question of the regeneration of ovarian tissue in the human subject. An appreciable number of cases have been reported (10, 15) where the removal of the ovaries in women, and even of part of the tubes as well, has been followed by pregnancy. In these cases the re-appearance of ovarian function has nearly always been put down to the incomplete removal of ovarian tissue. The results reported in the present paper show, we think conclusively, that careful surgical removal of all tissue which can reasonably be called ovarian, and even of the capsule and tube, may in the mouse be followed by the regeneration of gametogenic tissue. If such a process can occur in a rodent, it is not improbable that it may also occur in the human. In this case the occurrence of pregnancy after the removal of all ovarian tissue is not an impossibility, and many of the cases reported in the literature may be instances of actual regeneration of ovarian tissue from non-ovarian structures, rather than instances of hypertrophy of fragments of original ovary left behind at the time of operation.

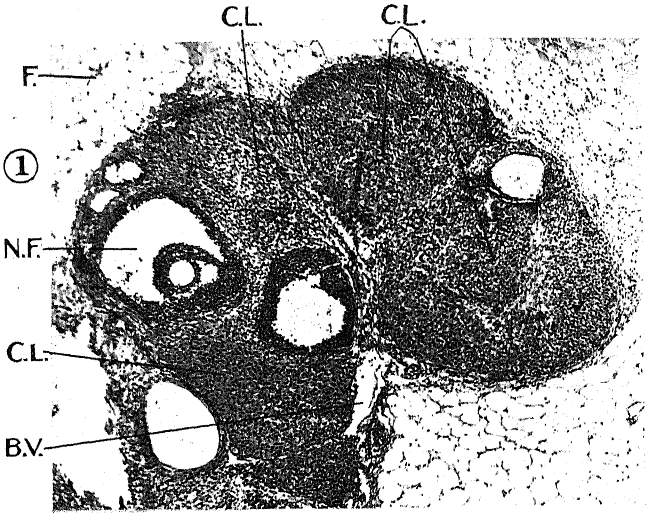
V. SUMMARY.

(1) Of 121 double ovariotomised mice, all of which showed initial cessation of the œstrous cycle, 11 subsequently showed signs of spontaneous œstrus. In eight of these 11 cases the presence of new ovarian tissue was demonstrated histologically.

(2) Since the operation of ovariotomy consisted of removing the ovaries, capsules and portions of the Fallopian tubes, it seems evident that the regeneration of ovarian tissue took place from non-ovarian structures, but the exact site of regeneration could not be ascertained from the material available.

(3) In most cases follicles and corpora lutea were found in the regenerate tissue, and in most cases also these cyclic structures of the regenerate tissue could be correlated with the recent œstrous history of the animal. In one or two cases, however, the fewness of these cyclic structures seemed to make it clear that the previous œstrous periods observed had not been brought about by the maturation of successive batches of follicles. This applies in particular to two animals which were sterilised by X-rays as soon as regeneration had begun.

(4) Most of the regenerate animals had a normal cycle after the first spontaneous appearance of œstrous symptoms, but in at least one case a very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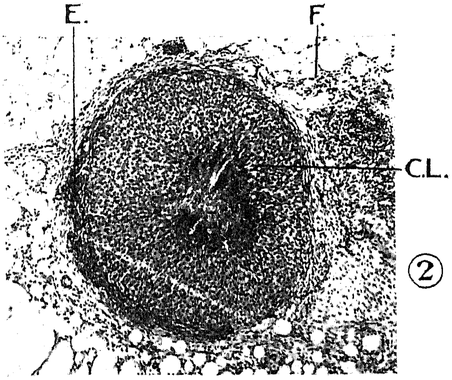
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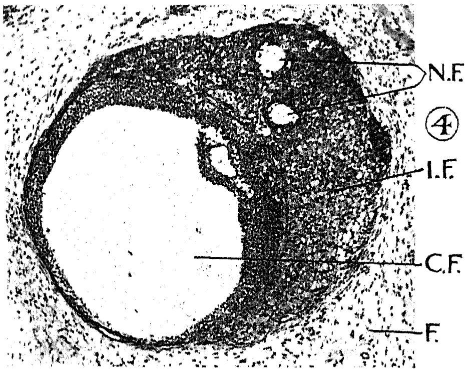
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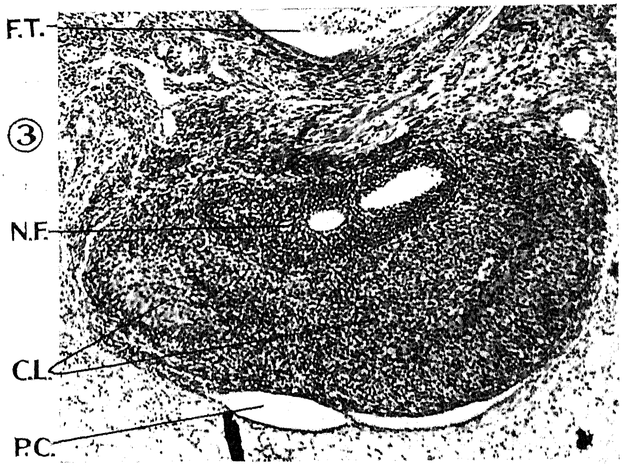
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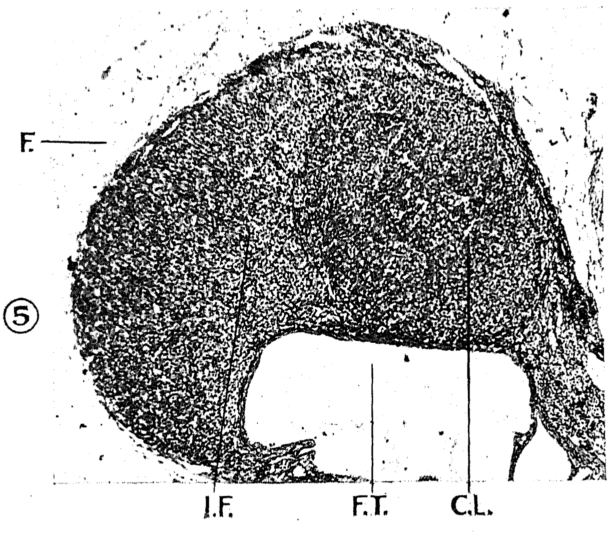
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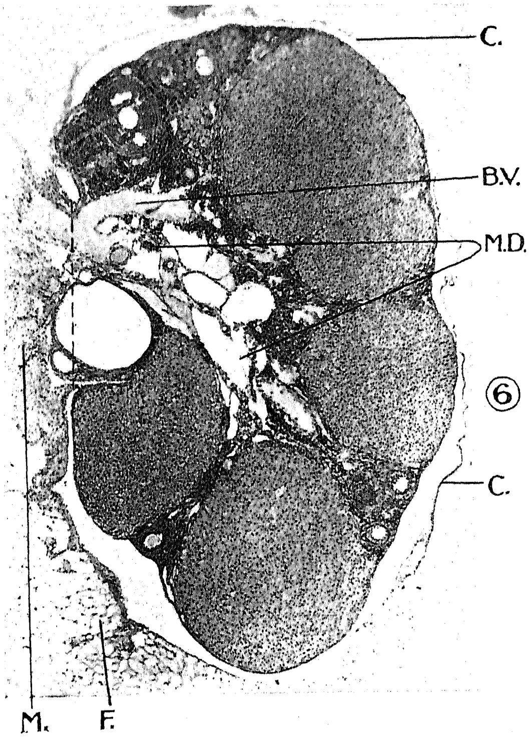
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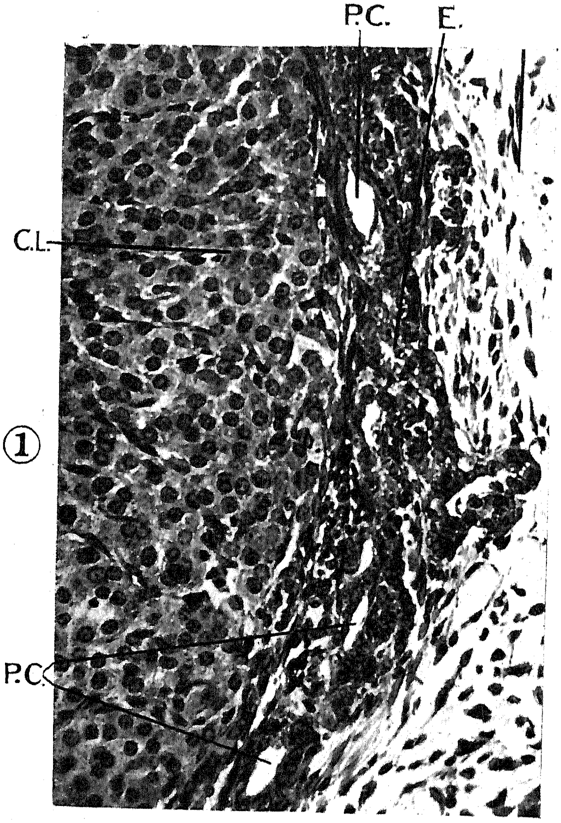

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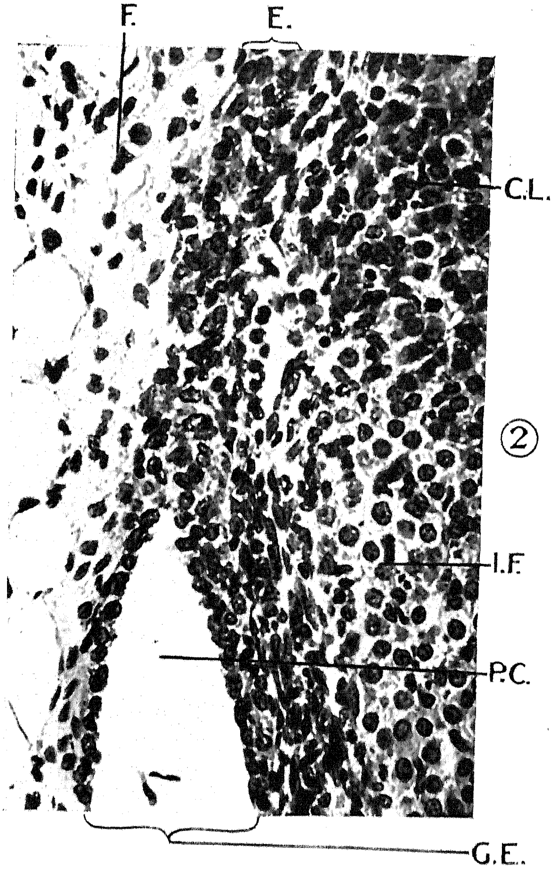

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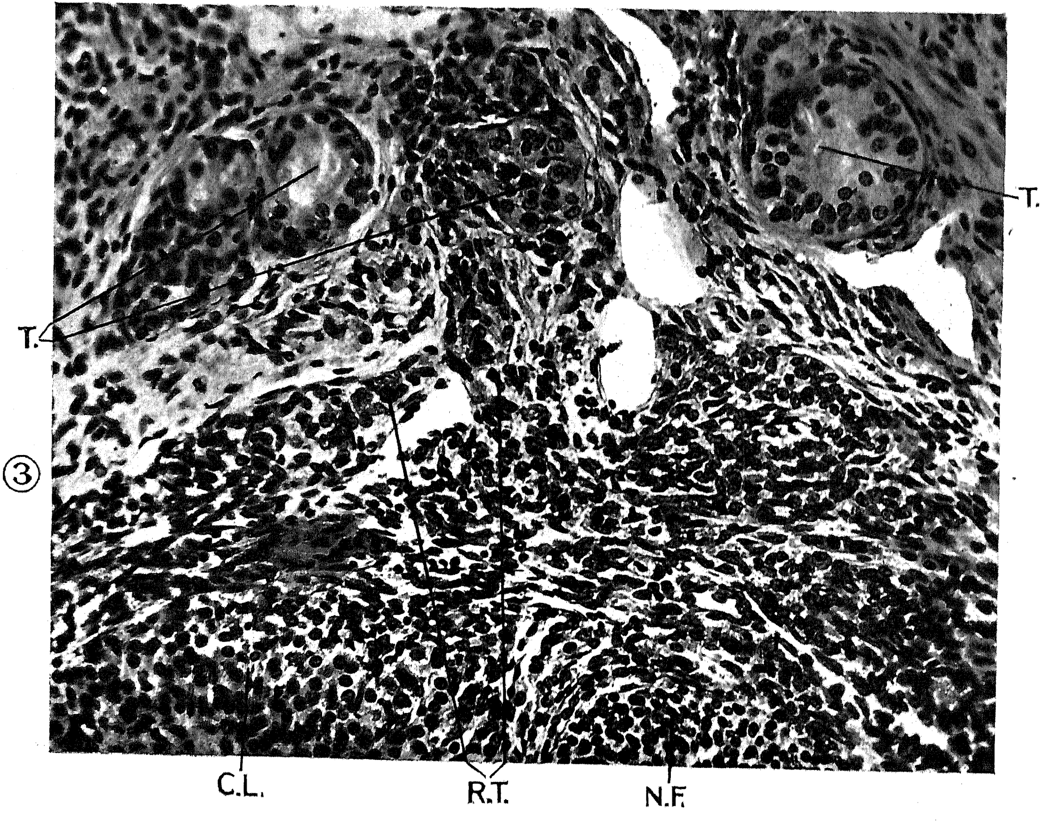

irregular series of œstrus-like phenomena occurred, prolonged cornification being the chief feature observed.

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DESCRIPTION OF PLATES 17 AND 18.

GUIDE LETTERS.—*B.V.*, Blood vessel. *C.*, Ovarian capsule. *C.F.*, Cystic follicle. *C.L.*, Corpus luteum. *E.*, Epithelial band. *F.*, Fat body. *F.T.*, Fallopian tube (cystic). *G.E.*, Epithelium lining periovarian cavity. *I.F.*, Interfollicular tissue. *M.*, Mesovarium. *M.D.*, Medulla. *N.F.*, Normal follicle. *P.C.*, Incipient periovarian cavity. *R.T.*, Rete-like connection. *T.*, Tubule.

PLATE 17.

FIG. 1.—OVS 9 showing corpora lutea and follicles in regenerate tissue. ×48.

FIG. 2.—OVS 13 showing corpus luteum, constituting almost all the regenerate tissue, with a narrow epithelial band around it. ×48.

FIG. 3.—OVS 8 showing two corpora lutea and one large flattened follicle in the regenerate tissue, which has an incipient periovarian cavity on one side. ×60.

FIG. 4.—OVS 52 showing one large cystic follicle and two small normal follicles in the regenerate tissue. ×48.

FIG. 5.—OVS 75 showing the relation of the cystic tube to the regenerate tissue. ×48.

FIG. 6.—OVS 117 showing one of the original ovaries removed at operation. The dotted line is drawn across the hilum. Note the intact capsule and the large amount of mesovarium attached to the ovary. ×38.

PLATE 18.

FIG. 1.—Portion of the periphery of the regenerate tissue in OVS 13 (Pl. 17, fig. 2) showing the narrow epithelial band containing traces of an incipient periovarian cavity. ×350.

Fig. 2.—Portion of the periphery of the regenerate tissue in OVS 8 (Pl. 17, fig. 3) showing the peripheral epithelial band dividing to form the epithelial lining of the incipient periovarian cavity. ×380.

Fig. 3.—Portion of the periphery of the regenerate tissue in OVS 8 (Pl. 17, fig. 3) showing rete-like connections between it and the tubules in the fat body. ×275.

We are indebted to Mr. F. J. Pittock for the photomicrographs reproduced in these plates.

The Giant Cells in the Placenta of the Rabbit.

By G. S. Sansom, D.Sc., Honorary Research Assistant in the Department of Embryology, University College, London.

(Communicated by Prof. J. P. Hill, F.R.S.—Received February 12, 1927.)

[Plates 19–27.]

Material and Methods.

The material on which this paper is based was collected partly by Prof. J. P. Hill and partly by myself. The uterine swellings with their contained embryos were fixed entire, either in Bouin's picro-formol-acetic mixture or in corrosive-sublimate-formol-acetic. The former gave good general fixation and penetrated well, even into the larger specimens. Tissues fixed in it were considerably easier to cut than those preserved in corrosive formol. In order to preserve the fœtal membranes as far as possible intact, it was desirable not to open the uterine swellings, except in the latest stages, where it was, of course, necessary; hence a considerable time was required for the penetration with paraffin.

It is very doubtful if a vacuum embedding bath is desirable for objects of this nature. Membranes may be ruptured unless the change in pressure is very gradual, hence I found it preferable to reduce the possibility of air bubbles forming in the interior of the uterus by employing air-free fixing solutions, followed by air-free alcohols of increasing strength. Clearing was carried out in cedar-wood oil and the tissues transferred direct to paraffin. Little trouble was experienced with air in the interior of the uteri, and this method of employing air-free solutions is well worth the slight extra trouble. If tissues are transferred from aqueous solutions into alcohol the air contained in the water is liberated in the tissues and cannot later be easily removed, since the stronger the alcohol the less air it is capable of dissolving.

I wish to express my indebtedness to Prof. J. P. Hill for his kind assistance and advice. I also wish to thank Mr. Pittock of the Institute of Anatomy for his valuable advice on photographic matters.

The photomicrographs which accompany this paper are untouched photographs, as I considered it more desirable to retain their evidential value than to improve their appearance pictorially.

General.

It is not proposed in this paper to describe the placentation of the rabbit. The already voluminous literature dealing with this subject has covered the ground very completely, and although the various authors are not in entire agreement, there are comparatively few points on which the more recent workers disagree. The very valuable detailed paper of Schoenfeld gives a remarkably clear account of the attachment of the blastocyst to the uterine wall and of the formation of the placenta. This paper was published in 1903, the same year that Chipman's long account appeared in the Laboratory Reports of the Royal College of Physicians of Edinburgh. Chipman appears to have had a very complete series of rabbit embryos fixed and cut *in utero*, but his photomicrographs are very difficult to interpret and afford little evidence in support of his statements. One of the main points on which he differs from Maximow and Schoenfeld is the formation of syncytiotrophoblast. He denies that any plasmodial change in the trophoblast occurs until the tenth day and states that the latter, before and during attachment to the uterine epithelium, is purely cellular in character. This statement it is impossible to accept, since in all well preserved specimens of 8 to 8½ days, the trophoblast is definitely differentiated into a cellular layer and a syncytial layer, even where fusion with the uterine epithelium has not yet taken place and where the latter is still a continuous, cellular, epithelial layer. (Plate 19, figs. 1 and 2.) Moreover, after fusion with the uterine epithelium has occurred, the nuclei of the maternal symplasma are clearly distinguishable from the more deeply staining nuclei of the syncytiotrophoblast. (Plate 20, fig. 3.)

Chipman states that Maximow, having postulated the formation of two syncytial layers, one maternal and one fœtal, finds a difficulty in disposing of the maternal syncytium, and considers his explanation artificial; but when one is confronted by a stage comparable to that illustrated in figs. 1, 2 and 3, where two syncytia, having rather different staining reactions, are undeniably present, there is very considerable difficulty in deriving them both from the uterine epithelium; moreover the uterine epithelium is intact in some places where the other syncytial layer is present (figs. 1 and 2). Therefore, if this syncytium is maternal in origin, it can only have been formed by the union with the cytotrophoblast of cells detached from the uterine epithelium, or at all events cells which have migrated from their sites of origin in the maternal tissues and passed out through the uterine epithelium or uterine glands. This alternative explanation is highly improbable, but, as a matter of fact, certain cells do appear to be budded-off from the maternal tissues, though apparently not in sufficient numbers to account for the amount of syncytiotrophoblast present. Moreover, there is very strong evidence that the syncytiotrophoblast is the direct product of the cytotrophoblast, for intermediate stages are fairly numerous. The significance of these budded-off cells, many of which appear to be degenerating (Plate 20, fig. 4), is not at present known, and apparently other writers have not described them. It is probable they are simply effete uterine epithelial cells in process of elimination.

First appearance of the Giant Cells.

In many rodents there appear in the uterine tissues during pregnancy conspicuous elements known as giant cells. These cells, which reach an enormous size, sometimes as much as 0·3 mm. in length, are usually mononucleate, but a certain number are multinucleate. They are present in great numbers in the walls of pregnant uteri of the rat, water-vole and rabbit, but are rarely found in the placental labyrinth. In the rat and water-vole they occur around the entire obplacental region of the uterine wall, but are most numerous in the region of the periplacental furrow which surrounds the placental disk. In the rabbit their distribution is less constant, some parts of the antimesometrial uterine wall being crowded with them and other parts almost devoid of them.

There is a great discrepancy in the works of previous writers as regards the first appearance of the giant cells. This is no doubt due partly to the fact that some of them based their conclusions on insufficient material of the requisite stages and partly to the difficulty of determining the precise origin of the structures themselves. Schoenfeld states that they appear on the seventh day when the disappearance or rupture of the zona allows the blastocyst to become attached to the uterine wall. The attachment, according to him, is actually conditioned by the penetration into the uterine wall of trophoblastic cells, or rather of the trophoblastic syncytial masses, which give origin to giant cells. If the giant cells are of fœtal origin it ought to be possible to determine the precise time of their first appearance, *e.g.*, in this case their appearance coincides with the attachment of the blastocyst; but where an author tries to derive them from maternal tissues there is difficulty in deciding when they arise. Chipman states that they appear on the sixth day, and shows a photomicrograph of a portion of the obplacental area in which numerous very large giant cells are present. Now this stage is prior to the attachment of the blastocyst to the uterine epithelium, hence these cells would appear to have been formed *in situ*, and, to judge by their size, I should say they must have been present at a considerably earlier date.

I have examined two series of sections through the uterine swellings at 6¾ days' gestation and found no trace of giant cells. In these series the blastocyst is present, and lies free in the uterine lumen and enclosed in its still intact zona. If, as Chipman maintains, fully formed giant cells are present in the obplacental uterine wall at six days' gestation, they must be of maternal origin, for the blastocyst is isolated from the uterine wall by its zona, and in the case Chipman cites the blastocyst was not even present. What then is the explanation of the presence of these cells if one does not accord them a maternal origin ? The most probable explanation, if one excepts the possibility of the stage in question being much older than Chipman thought, is that these giant cells had persisted from a previous pregnancy and that the uterus was *post-partum* instead of 6-days' gestation.

From my own observations I am led to believe that the majority of the giant cells in the uterus of the rabbit are of fœtal origin, and that only in the later stages are any formed from the maternal tissues.

At 6½-days' gestation the blastocyst lies free in the uterine cavity and is still invested in its zona, which, however, shows signs of disappearing. The blastocyst is orientated in its definitive position, with the embryonal area adjacent to the placental ridges of the uterine mucosa. As Schoenfeld has described, there are present, at various places on the antimesometrial hemisphere, localised trophoblastic multinucleate thickenings, which represent the predetermined areas for attachment to the uterine epithelium. The latter consists of a very definite layer of columnar cells with ovoid nuclei. The vascularity of the mucosa is very marked, particularly in the periplacental region, where the folds appear as if gorged with blood. The endothelial cells lining the superficial capillaries tend to stain very deeply, and, in some places, the endothelial cells seem to pass between the uterine epithelial cells so as to reach the surface of the mucosa. (Plate 20, fig. 5.)

During the course of the 7th day the wall of the blastocyst, freed from its zona, comes into contact with the uterine epithelium, and the multinucleate trophoblastic thickenings fuse with the latter, thus attaching the blastocyst around its antimesometrial hemisphere. The embryonal area is still free from, though in close apposition to, the placental ridges of the mucosa. Towards the close of the 7th day the uterine epithelium in the obplacental region shows marked changes. The epithelial nuclei multiply and the cytoplasm undergoes great hypertrophy. Cell outlines disappear and the numerous nuclei become crowded together in the middle region of the cytoplasm which exhibits a differentiation into two zones : (i) a deeper, dark staining, finely granular zone in continuity with the epithelium of the uterine glands, and (ii) a superficial, pale-staining zone having a finely reticular or foam-like structure.

The epithelial nuclei are situated in the inner zone of more deeply staining cytoplasm and define its outer limits. The more deeply situated portions of the uterine glands remain unchanged, but the superficial parts undergo changes comparable to those in the uterine epithelium. The trophoblastic wall of the blastocyst has in places fused with the above-mentioned outer cytoplasmic zone, and its nuclei appear embedded in it (Plate 21, figs. 6 and 7). At certain places, where the multinucleate trophoblastic masses are present, the uterine epithelium is destroyed and these multinucleate bodies may be seen passing into the corium (Plate 22, figs. 8 and 9), where they increase in size and form the giant cells of the obplacental region. These giant cells are active phagocytic bodies of irregular shape, their cytoplasm being often drawn out into long pseudopodial like processes. Their nuclei are large and contain dark chromatin granules. Where they have passed through the uterine epithelium and reached the sub-epithelial capillaries the walls of the latter are destroyed and the red blood corpuscles engulfed.

At about this time connection is also established with the two periplacental ridges through the agency of well-marked trophoblastic multinucleate swellings. The maternal tissues at these periplacental sites of attachment seem particularly active, the folds being exceedingly vascular. One often finds at this stage a band of deeply staining spindle-shaped cells, clothing the wall of the blastocyst where it bridges over the furrow, between the periplacental attachment and adjacent obplacental attachment. These spindle-shaped cells are quite distinct from the trophoblast cells of the vesicle wall with which they are in contact. It is probable that they are degenerating uterine epithelial cells, but red blood corpuscles are present amongst them and it is possible that endothelial cells of the superficial capillaries, which have been opened up, are also present. The significance of this is not clear to me, but it is noteworthy that it occurs in the region where the giant cells of the late placenta take their origin, and where the trophoblast is especially active (Plate 22, fig. 10).

During the 8th day, when the attachment of the blastocyst to the placental ridges takes place, there is a progressive destruction of the uterine epithelium and uterine glands of the obplacental region, under the influence of the trophoblast. The uterine epithelial symplasma becomes broken up into masses of vacuolated cytoplasm containing deeply staining pycnotic nuclei. The invading trophoblast cells are clearly recognisable, but although many of them have increased in size they are not remarkably large, incomparably smaller than those figured by Chipman at 6-days' gestation. There is a considerable thinning of the uterine wall at this time, especially around the antimesometrial pole. This is due partly to the destruction of the uterine epithelium and glands and partly to stretching by the expansion of the fluid-filled blastocyst. In the placental region most important changes are taking place. The syncytiotrophoblast is formed and invades the uterine symplasma, in the manner so clearly described and figured by Maximow and Schoenfeld and referred to above (Maximow's figs. 1 and 2 and Schoenfeld's fig. 10).

By the middle of the 9th day the uterine epithelium at the antimesometrial pole has almost disappeared, together with the uterine glands. The epithelial nuclei are recognisable as masses of darkly staining pycnotic nuclei overlying the connective tissue of the mucosa. The latter is in many places so thin that these degenerating nuclei are almost in contact with the layer of circular muscle. The trophoblastic giant cells are more clearly recognisable now, owing to their increase in size, but they are not very numerous around the antimesometrial wall. That part of the trophoblast just outside the periplacental furrow is proliferating rapidly, the multinucleate masses thus formed passing into the maternal symplasma and becoming giant cells. It is this part of the vesicle wall which provides the bulk of the giant cells present in the later stages. Maximow drew attention to the proliferation of the trophoblast in the periplacental region, and stated that its cells became giant cells which invaded the maternal tissues, and his fig. 12 is a true representation of the condition which obtains; yet other workers appear to have ignored this evidence in favour of the trophoblastic origin of these structures.

Although the destruction of the uterine epithelium is initiated by the penetration therein of the multinucleate trophoblastic masses, it apparently continues independently of their presence, or rather the extent of the destruction does not seem to be proportional to the number of giant cells present in the tissues. By the end of the 9th day for instance, the uterine and glandular epithelia over some parts of the antimesometrial wall have disappeared, the uterine wall there consisting of the two muscle coats, a very thin connective tissue layer and a membrane composed of a single layer of cubical cells derived from the more deeply situated portions of the uterine glands which have escaped destruction. This layer is in fact the regenerated uterine epithelium, which, from now on, persists throughout gestation, in spite of the fact that numerous giant cells come into contact with it and pass through it into the underlying tissues. Comparatively few giant cells are to be found in the antimesometrial uterine wall at this period, but they occur in large numbers in the masses of detritus in the uterine cavity. In the periplacental region, on the other hand, many of the uterine glands persist unchanged throughout gestation and yet the trophoblast is here proliferating very rapidly.

The explanation of this is to be found in the migratory nature of the giant cells, and also in the varying susceptibility of the tissues in different regions to the attack of these cells. The absence of large numbers of giant cells from the antimesometrial uterine wall is probably largely due to the very rapid destruction and sloughing-off of the superficial layers. The tissues into which they have penetrated have liquefied and come away from the uterine wall, carrying the giant cells with them. By the 10th day, indeed, the antimesometrial wall has reached its thinnest condition. The muscle layers are immune against destruction, and although, during the latter half of pregnancy, giant cells are found embedded in both longitudinal and circular muscle coats they do not appear to have any destructive action on them.

From the 10th day until the 25th the giant cells are conspicuous objects in the obplacental uterine wall. They attain their maximum size and number at about the 17th day when they occupy more than half the thickness of the wall (Plate 23, fig. 12). A certain number of them have arisen *in situ* by subdivision of the earlier invading cells (figs. 13 and 14), but there is comparatively little indication of giant cell division at this time (17 days) and the majority have certainly migrated to their present positions from the thickened layer of trophoblast in the region of the periplacental ridges. This layer of trophoblast I propose to speak of, henceforth, as the "trophoblastic fringe," but it should be noted that it consists not only of trophoblast but also of an underlying layer of entoderm.

The entoderm of the bilaminar omphalopleure, it may be mentioned, persists after the more or less complete disappearance of the trophoblast, as a thin membrane forming the antimesometrial wall of the yolk sac cavity. It is distinguishable up to about the 14th day; at about that time it breaks down, with the result that the yolk sac splanchnopleure comes into apposition with the uterine wall (Plate 24, fig. 15). In the earlier stages of development, the antimesometrial hemisphere of the blastocyst is formed by the bilaminar omphalopleure, which near the placental margin is in continuity on the one hand with the chorion læve, passing to join the placental margin, and on the other with the yolk sac splanchnopleure. The trophoblast over the greater part of the bilaminar omphalopleure fuses with the uterine wall and disappears as a continuous layer. Close to the junction with the chorion læve and yolk sac splanchnopleure, however, the trophoblast persists as a thickened layer of actively proliferating cells—the trophoblastic fringe.

The trophoblastic fringe is sometimes found with its distal edge free in the uterine cavity, as in fig. 16, but usually that edge fuses with the uterine wall and remains attached thereto until about the 17th day (Plate 25, fig. 17). Its cells reach a large size and are budded off, either singly or more often as compact groups or "cell islands" into the uterine cavity. They become attached to the regenerated uterine epithelium, pass through it and come to rest in the underlying tissues. A considerable number of these cells pass directly into the mucosa where it is in contact with the trophoblastic fringe. These cell islands (fig. 18) are composed of large cells with distinct cell outlines and one or two deeply staining nuclei rich in chromatin. Their cytoplasm which stains deeply with eosin has a finely granular or reticular structure. They are very conspicuous objects in the uterine cavity at 12-days' gestation, but later their place is taken by other structures, of a quite different appearance, which are also products of the fœtal trophoblast in the periplacental region, but which arise by a process of budding from the chorion læve.

Reference to Plate 26, figs. 19 and 20, will explain the relationship of the fœtal membranes in this region at 17-days' gestation. The membrane covered by the layer of tall columnar entoderm cells thrown into folds and villi, occupied by fœtal capillaries, is the yolk sac splanchnopleure. At the junction of this membrane with the chorion its entoderm is reflected back as a thin layer of fusiform cells in contact with the trophoblast; it has, in fact, little changed since its first appearance. Around the antimesometrial hemisphere it is no longer present as a continuous membrane. The trophoblastic fringe is still clearly recognisable, being composed of cubical cells proximally and of large ovoid or spherical cells distally. The distal cells are identical in character with those of the cell islands to which they have given birth; they are, however, less actively proliferative at this time (17 days) and some of the cells show degenerative changes. The proximal continuation of this trophoblastic layer forms the trophoblast of the chorion læve. It is that part of the original trophoblastic wall of the vesicle which has neither taken part in the formation of the placental labyrinth nor fused with the uterine wall. A portion of it occurs in the interplacental furrow.

As the placental ridges increase in size, the circum- or periplacental furrow deepens and the chorion læve creeps up the margins of the placental disk thus forming the inner wall of the circumplacental furrow. It consists of two layers, (*a*) trophoblast and (*b*) mesoderm. The trophoblast has the form of a single layer of densely crowded cubical cells, many of them in a state of very active mitosis. The cytoplasm stains intensely with eosin, the nuclei are exceedingly rich in chromatin and mostly spherical. From this layer there are budded-off into the uterine cavity great numbers of multinucleate spheres, which have a characteristic form (Plate 27, fig. 21). They consist of spherical masses of cytoplasm crowded with densely staining nuclei, similar to those of the chorion læve. The nuclei are arranged either peripherally, close to the surface of the cytoplasm, or are aggregated into spherical masses, occupying in some cases the central region of the cytoplasm, in others placed excentrically. The cytoplasm of these multinucleate spheres is at first similar to that of the parent cells, but it soon becomes coarsely reticular and in that part where the nuclei are aggregated it stains very faintly.

These bodies occur in great numbers in the uterine cavity, between the yolk sac splanchnopleure and the regenerated uterine epithelium, and also in the interplacental furrow, where a similarly modified portion of the trophoblast is to be found. Unlike the giant cells these multinucleate spheres are not active ; they lie free in the uterine cavity amongst the detritus formed by the degenerating uterine epithelium and may be seen in all stages of decay. A very considerable destruction of maternal tissues, uterine epithelium and connective tissue takes place in the circumplacental furrow at this time, and there is little doubt that this destruction is brought about by the trophoblast of the chorion læve, possibly with the object of reducing the area of attachment of the placental disk to the uterine wall preparatory to parturition. Although there is no doubt that the majority of these multinucleate spheres are budded-off from the chorion læve, it is probable that some of them contain nuclei derived from the degenerating uterine epithelium.

We find therefore a marked change in the seat and character of trophoblastic activity in the periplacental region after about the 15th day. The trophoblastic fringe, from the time of its appearance as a definite localised thickening of the vesicle wall at nine days until the 14th day, exhibits proliferative activity. Its cells enlarge, become detached, penetrate the uterine epithelium and pass into the corium of the mucosa, where they grow to an enormous size and may be found in large numbers in the muscle layers. After the 15th day, however, there is a reduction in the activity of this layer, and by the 17th day it has apparently ceased to proliferate. The trophoblast of the chorion læve, on the other hand, is almost inactive until the 14th day and reaches its maximum activity about the 21st day. The multinucleate spheres, to which it gives origin, do not possess the power to penetrate the uterine epithelium, hence they are never found in the mucosa. Their nuclei become structureless, intensely black-staining bodies, which soon break up into smaller fragments and ultimately disappear. The outer cytoplasmic walls sometimes persist after the disappearance of their contents.

The Origin of Giant Cells from Maternal Tissues.

According to Duval, Minot and Chipman, the giant cells of the rabbit are all products of maternal tissues. Duval derives them from the connective tissue cells; Minot and Chipman from uterine epithelial cells; Schoenfeld, on the other hand, considers that they are purely trophoblastic in origin, and my own observations leave me with little doubt that his view is correct, in so far as it applies to the large giant cells of the peri- and obplacental regions; but Schoenfeld was not concerned with the later stages of pregnancy, and it is during the latter half of gestation that cells, having the character of giant cells, appear in the deeper tissues of the placental ridges.

The careful observations of Maximow led him to believe that a certain number of giant cells were formed from the periendothelial elements (the "perithel" layer of Maximow), or from the endothelial cells themselves, and there is very strong evidence in support of this view.

If one examines the deeper placental region at 12 days one finds the endothelial cells of the maternal capillaries in a state of very active proliferation (fig. 22). The cells, which are large and ovoid, with conspicuous nuclei, form in-bulgings into the lumina of the capillaries. Many of the nuclei are to be seen in stages of mitosis. It is difficult to believe that these cells are trophoblast cells which have destroyed and replaced the endothelium of the capillaries, for they are found in vessels close to the mesometrium, far from the deepest limit of the placental labyrinth. The fact that they are dividing mitotically also militates against the view that they are trophoblastic. It is equally difficult to believe that they are glycogen cells, to which they bear no resemblance. It seems extremely probable that they are greatly hypertrophied endothelial cells, such as occur in the placenta of the rat and water-vole. According to Maximow, a certain number of these cells become detached from the endothelium and are carried in the blood stream to other parts of the uterine wall. He regards this as the normal procedure, but I think it is more probably accidental. Some of these hypertrophied cells form bridges across the lumina of the capillaries and it is quite possible that they might break away from their attachment to the adjoining cells and come to lie free in the blood stream.

This endothelial proliferation increases as gestation proceeds, but owing to the increase in size of the placenta it becomes more and more confined to the region close to the mesometrium. Meanwhile, the enormous giant cells of the obplacenta gradually disappear. Many of them fragment, forming a number of smaller uni- or multi-nucleate cells, which are quite inconspicuous and apparently inactive. A certain number of the large cells, especially those situated in the circular muscle coat, seem to undergo absorption without fragmentation; the cell body becomes very thin and attentuated and is difficult to distinguish from the muscle fibres between which it is compressed.

By the 25th day of gestation there are no large obplacental giant cells remaining, but some of the capillaries in this region are surrounded by many small giant cells, identical in appearance with those around the deep placental capillaries. In most cases the endothelial walls of these vessels are intact, and but little hypertrophied, the giant cells forming a sheath around them, but in others the vessel walls appear to be formed in part by these cells.

The question arises as to the origin of these small obplacental giant cells. There are three possibilities :—

(i) They may be the daughter cells of the original obplacental giant cells and therefore of fœtal origin.

(ii) They may be maternal endothelial cells which have migrated in the blood stream, as Maximow suggests, or through the muscle layers, to the obplacenta from their seat of origin in the mesometrial region.

(iii) They may be formed from the obplacental endothelial cells, or connective cells.

We may consider these three possibilities in order.

(i) In the obplacenta at 21 days' gestation there are present a number of very large giant cells, some of which exhibit signs of impending division by amitosis. By the 25th day no large cells remain, but some groups of smaller cells are recognisable which are obviously the products of larger cells, and they are identical in appearance with the cells aggregated around the capillaries (fig. 24). It is certain, therefore, that some at least of these small giant cells are trophoblastic elements.

(ii) If the maternal endothelial cells migrate to the obplacenta *via* the lumen of the capillaries, one would expect to find very many of them free in the blood stream, but, as a matter of fact, the number of free-swimming cells is exceedingly small. Very rarely a free cell may be found, but, as already mentioned, this is probably accidental, since the hypertrophied endothelial cells project into the lumina of the capillaries and their area of attachment to the vessel walls becomes greatly reduced compared with their bulk. A serious objection to their transportation in this manner is the risk of their causing a stoppage of the blood stream in the smaller vessels, and the possibility of their being carried to other regions than the uterine wall. As to whether they migrate through the muscle layers or not is another matter, and one which is very difficult to determine, but I have seen no indications of their migratory character.

(iii) If these elements are formed *in situ* by proliferation of the endothelial cells of the obplacental capillaries, one would expect to find these latter in a state of active proliferation and hypertrophy, as is observable in the mesometrial region, but here the capillary walls are in most cases intact and of normal appearance, the giant cells forming sheaths around them. I can find no evidence of their origin from connective tissue cells.

It seems highly probable, therefore, that these small obplacental giant cells are all remnants of the great trophoblastic elements, and that their accumulation around the capillaries has some other significance. It should be clearly understood that although many of the endothelial cells of the deep placental capillaries undergo great hypertrophy and exhibit proliferative activity, they never attain to the dimensions of the obplacental trophoblastic giant cells. Compared with the latter, they are inconspicuous. Their greatest length is about 0·1 mm., whereas the trophoblastic giant cells sometimes reach a length of 0·4 mm.

These endothelial giant cells are confined to the immediate neighbourhood of the capillaries. They attain their maximum size by the 21st day, after that fragmentation of the nuclei sets in and about the 25th day many of them are to be seen undergoing degeneration.

Summary.

In the pregnant uterus of the rabbit, two kinds of giant cells are found. The larger and most conspicuous variety is derived from the fœtal trophoblast, cells from which become detached from the blastocyst, about the 7th day of gestation and penetrate into the obplacental mucosa. These cells grow rapidly and attain an enormous size, sometimes as much as 0·4 mm. in length. They persist until about the 22nd day, when fragmentation sets in and they become.

broken up into comparatively small bodies. Large numbers of these cells are also formed from that portion of the trophoblast of the proximal zone of the bilaminar omphalopleure, which projects free into the uterine cavity after the attachment of the blastocyst to the placental folds on the 8th day and the disappearance of the remainder of the omphalopleure. The cells proliferated off from this "trophoblastic fringe," pass into the uterine cavity, penetrate the regenerated uterine epithelium of the antimesometrial uterine wall and enter the underlying tissues. The proliferation of this "trophoblastic fringe" persists until the 16th day of gestation.

The mesometrial giant cells are of maternal origin, being formed by the proliferation of the endothelial lining of the capillaries in the deep placental region. They appear at about the 11th day of gestation and persist until after the 27th day. They never attain a great size and are confined to the mesometrial region. The trophoblast of the chorion læve gives origin to great numbers of multinucleate spheres, which become free in the uterine cavity and in the inter-placental furrow. These bodies are inactive degenerate structures.

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DESCRIPTION OF PLATES 19 to 27.

KEY TO ABBREVIATIONS.

Bl. C., Red Blood corpuscle.
Bl. W., Wall of blastocyst.
Bi. Om., Bilaminar Omphalopleure.
Ch. L., Chorion læve.
Cy. Tr., Cytotrophoblast.
E.C., Extra-embryonal cœlom.
End. C., Endothelial cell.
Ent., Entoderm.
F. C., Fœtal capillary.
G. C., Giant cell.
M. C., Maternal capillary.
M. S., Multinucleate sphere.
Syn. Tr., Syncytiotrophoblast.
Tr., Trophoblast.
Tr. Ch. L., Trophoblast of Chorion læve.
Tr. F., Trophoblastic fringe.
Tr. N., Trophoblastic nucleus.
Tr. M., Trophoblastic multinucleate mass.
Ut. C., Uterine cavity.
Ut. Ep., Uterine epithelium.
Ut. Gl., Uterine gland.
Ut. Sym., Uterine symplasma.
Ut. W., Uterine wall.
Ys. C., Yolk sac cavity.
Ys. Sp., Yolk sac splanchnopleure.

PLATE 19.

FIG. 1.—Transverse section through a portion of the placental lobe at about 8 days, showing the attachment of the blastocyst to the uterine epithelium of the placental ridges, and the formation of syncytiotrophoblast from the cytotrophoblast. The uterine epithelium has, in places, become converted into a symplasma, into which the darkly stained nuclei of the syncytiotrophoblast have penetrated. × 190.

FIG. 2.—A section similar to that shown in fig. 1, showing the formation of syncytiotrophoblast from the cytotrophoblast. The greater part of the uterine epithelium is still unchanged. × 200.

PLATE 20.

FIG. 3.—A high-power view of the region shown in fig. 1. The formation of the uterine symplasma by the epithelium of the uterine glands and its invasion by the large trophoblastic nuclei. × 430.

FIG. 4.—Part of the placental ridge at about 8 days, showing the budding-off of cells from the uterine epithelium. × 430.

FIG. 5.—Part of the placental ridge at 8 days, showing the penetration of endothelial cells into the uterine epithelium. × 340.

PLATE 21.

FIG. 6.—Transverse section through a portion of the uterine wall at 8 days, close to the periplacental region, showing the attachment of the blastocyst in the obplacental region. The uterine epithelium has become converted into a symplasma with the nuclei crowded into the central region. The trophoblastic nuclei are penetrating into this symplasma. The thin membrane overlying the symplasma is the entoderm of the yolk sac wall. × 140.

FIG. 7.—A high-power view of this region, showing the maternal symplasma containing a few trophoblastic nuclei. × 300.

PLATE 22.

FIG. 8.—Part of the obplacental uterine wall at 9 days, showing the earliest appearance of the giant cells. The trophoblast multinucleate masses cause local destruction of uterine epithelium symplasma, pass through the gaps so formed and become giant cells. × 180.

FIG. 9.—Part of the obplacental uterine wall at 9 days, showing two newly formed giant cells, lying in the corium just below the masses of pycnotic nuclei formed by the degenerating uterine symplasma. × 190.

FIG. 10.—The attachment of a multinucleate trophoblastic mass to the periplacental ridge at about 7¾ days. The uterine epithelium over the area of contact has disappeared, and the wall of a superficial capillary destroyed. The red blood corpuscles may be seen engulfed by the trophoblast. × 275.

FIG. 11.—Part of the uterine wall in the periplacental region. The actively proliferating band of trophoblast gives origin to the majority of the giant cells. In some places the uterine epithelium has entirely disappeared, in others it is represented by the layer of symplasma with crowded pycnotic nuclei. × 100.

PLATE 23.

FIG. 12.—The uterine wall in the obplacental region at 17 days' gestation. Giant cells are present in great numbers even in the muscle layers. The regenerated uterine epithelium is formed from the basal portions of the uterine glands. × 150.

FIGS. 13 AND 14.—Parts of the antimesometrial uterine wall at 12 days' gestation, showing giant cells in process of division by amitosis. × 170 and 155.

PLATE 24.

FIG. 15.—The antimesometrial uterine wall and the yolk sac splanchnopleure at 21 days' gestation. The original entodermal wall of the blastocyst has disappeared. In the uterine cavity are numerous multinucleate spheres derived from the trophoblast of the chorion læve. × 130.

FIG. 16.—Transverse section through the periplacental region of the uterus at 12 days' gestation. The trophoblast of the bilaminar omphalopleure is in a state of active proliferation and constitutes the "trophoblastic fringe," which gives origin to the majority of the giant cells. × 30.

PLATE 25.

FIG. 17.—Transverse section through the uterine wall in the peri-placental region at 12 days' gestation. The "trophoblastic fringe" is here fused to the uterine wall and the giant cells composing it are invading the maternal tissues. Remnants of the uterine epithelial symplasma are still present. × 190.

FIG. 18.—A cell island in the uterine cavity detached from the trophoblastic fringe. × 170.

PLATE 26.

FIG. 19.—Transverse section through the periplacental region of the uterus at 17 days' gestation showing the arrangement of the fœtal membranes. × 30.

FIG. 20.—A high-power view of the same region as that shown in fig. 19, to show the relationship of the bilaminar omphalopleure (trophoblastic fringe), chorion læve and yolk sac splanchnopleure. The trophoblast of the chorion læve is budding-off multinucleate spheres. × 170.

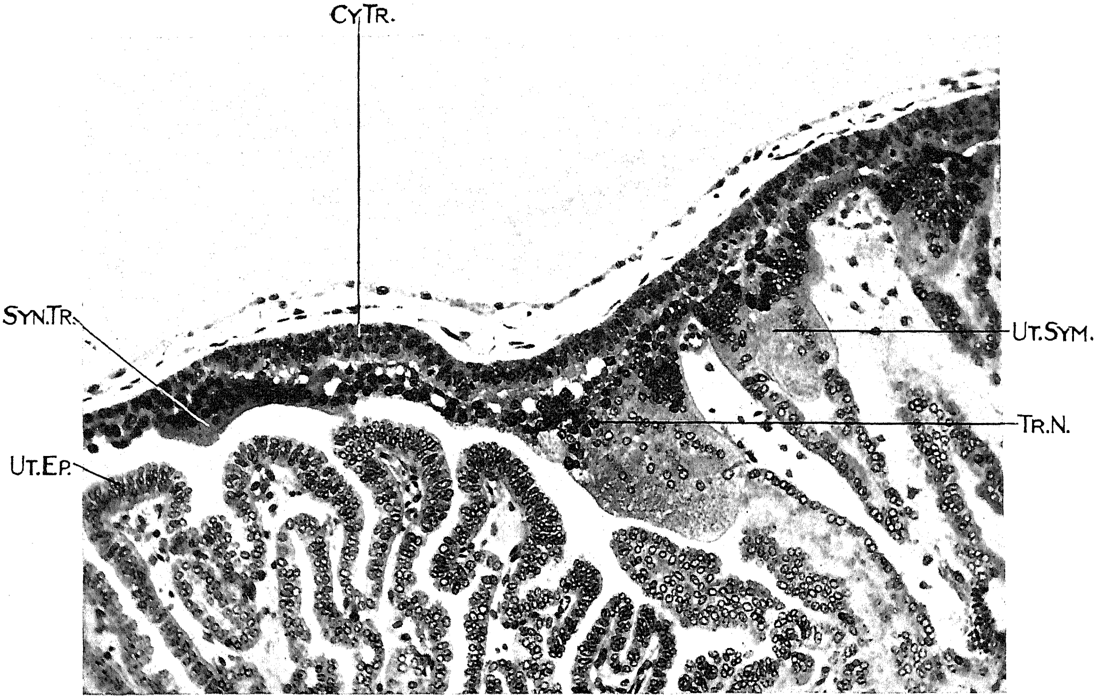
PLATE 27.

FIG. 21.—A portion of the chorion læve and the multinucleate spheres which are budded-off from it; 21 days' gestation. × 340.

FIG. 22.—A capillary in the deep placental region at 12 days' gestation. The endothelial cells are in active mitosis and greatly hypertrophied. × 155.

FIG. 23.—A capillary in the obplacental region of the uterine wall at 12 days' gestation. Two giant cells are apposed to the endothelial wall, but they are of fœtal trophoblastic origin. × 325.

FIG. 24.—A part of the antimesometrial uterine wall at 25 days' gestation. The small giant cells are formed by fragmentation of the large trophoblastic elements. They show a tendency to accumulate around the capillaries. × 95.



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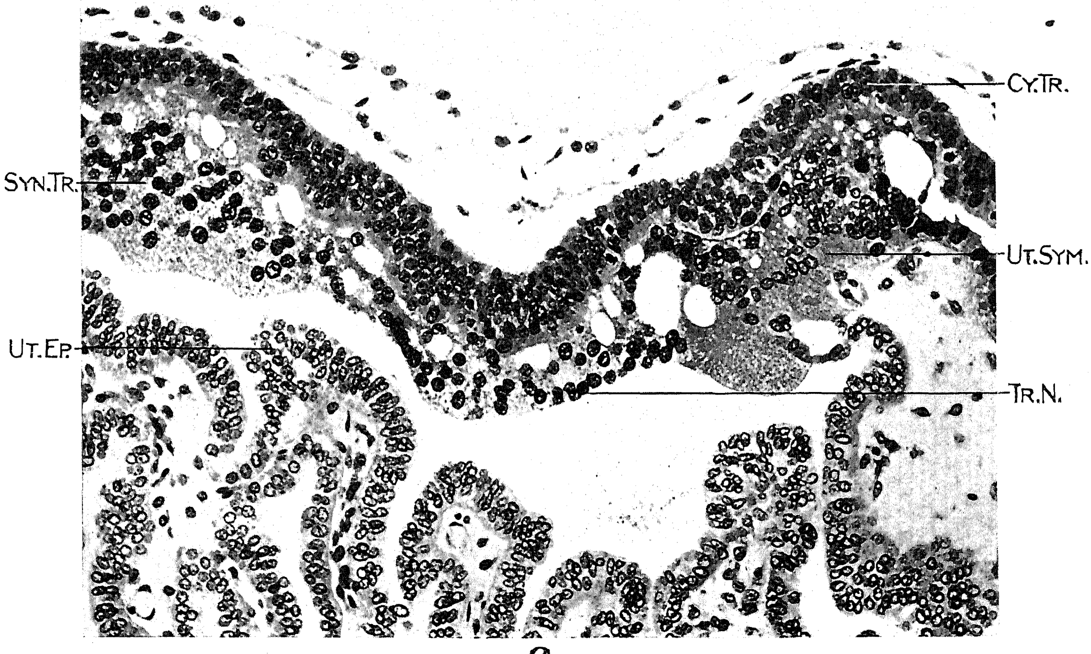

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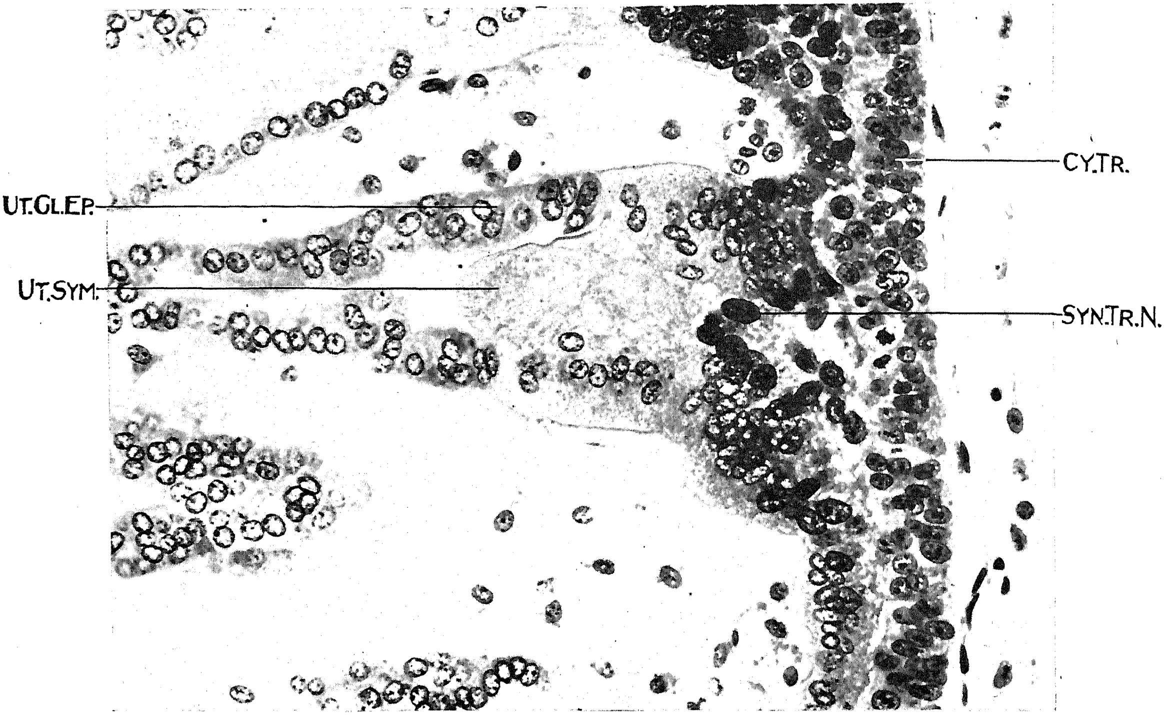

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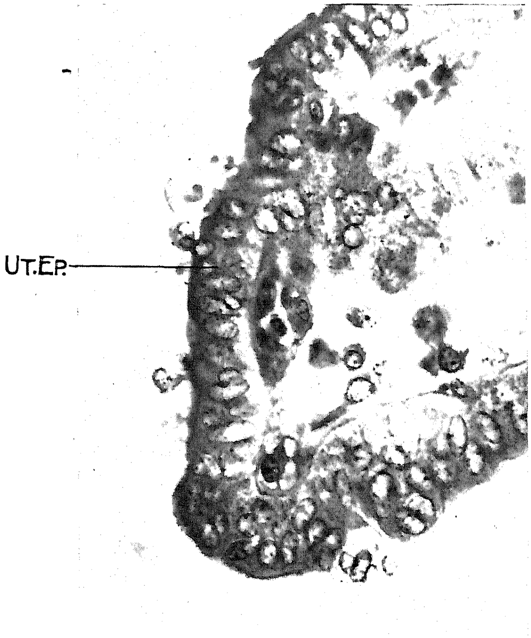

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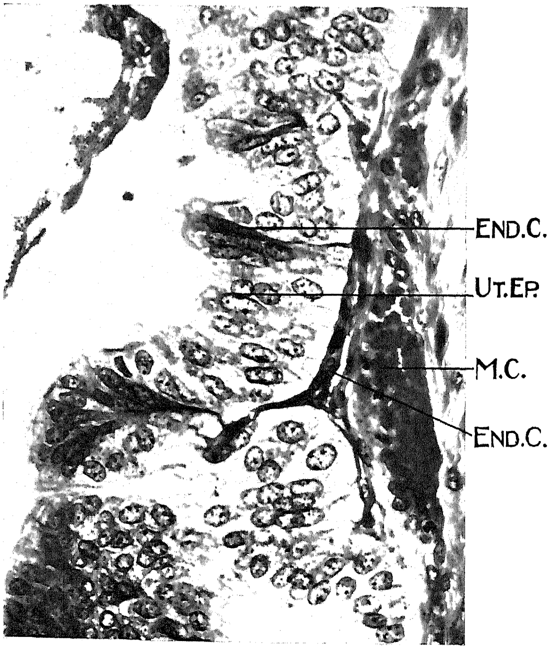

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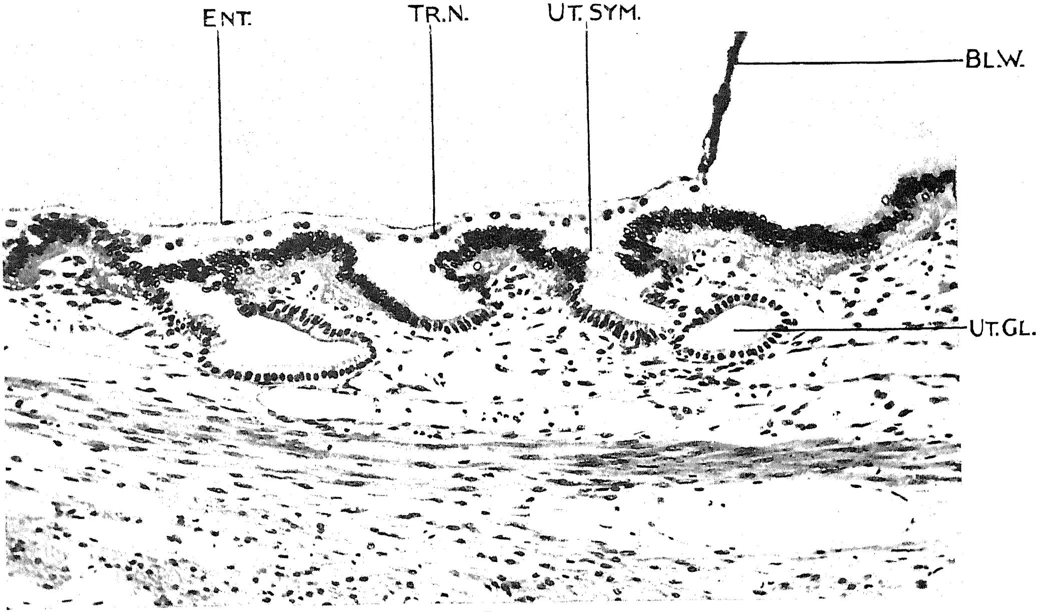

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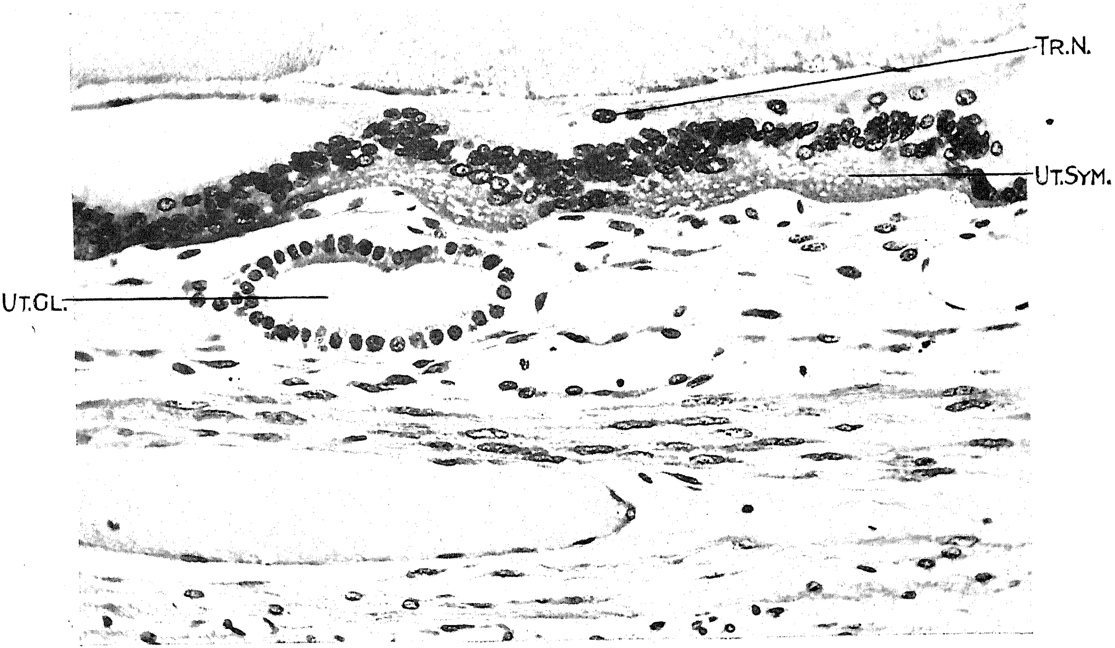

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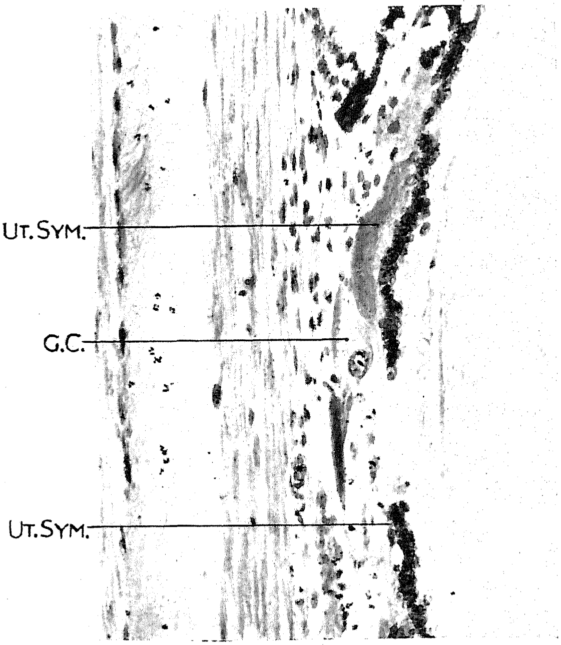
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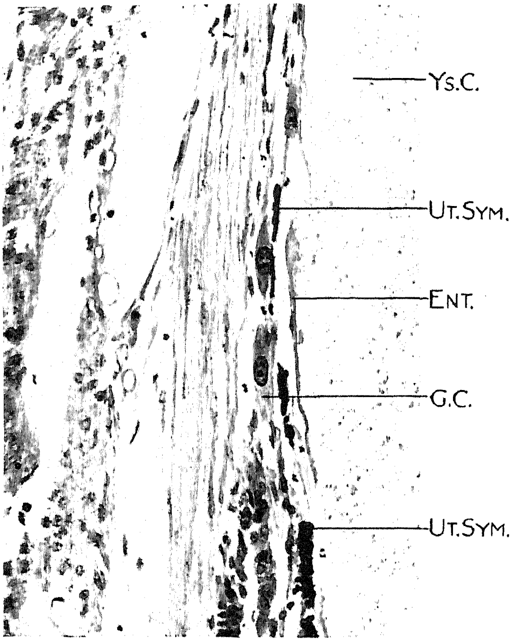
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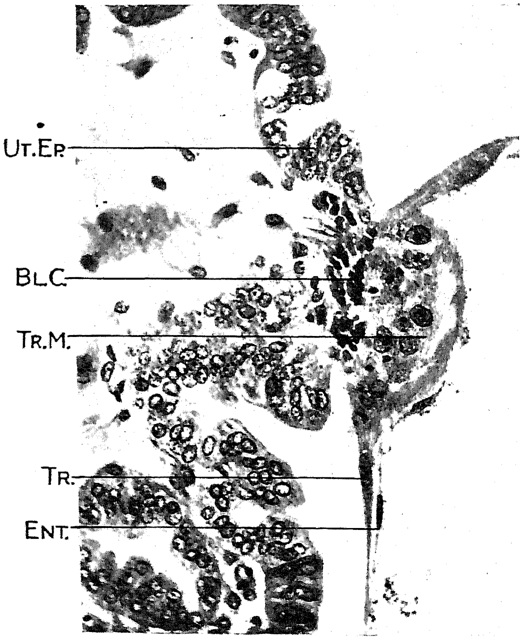
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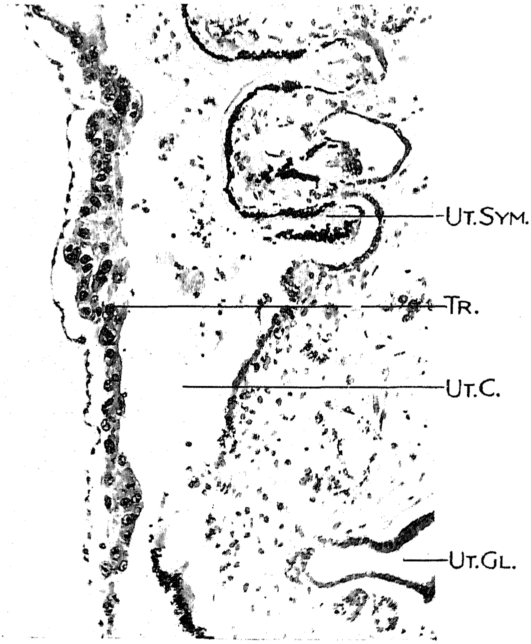
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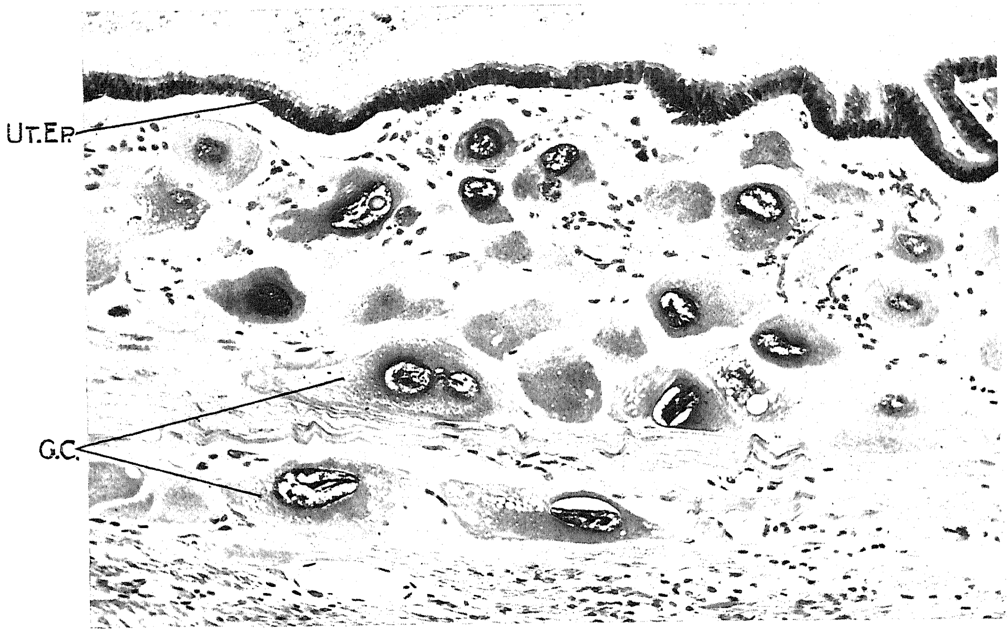

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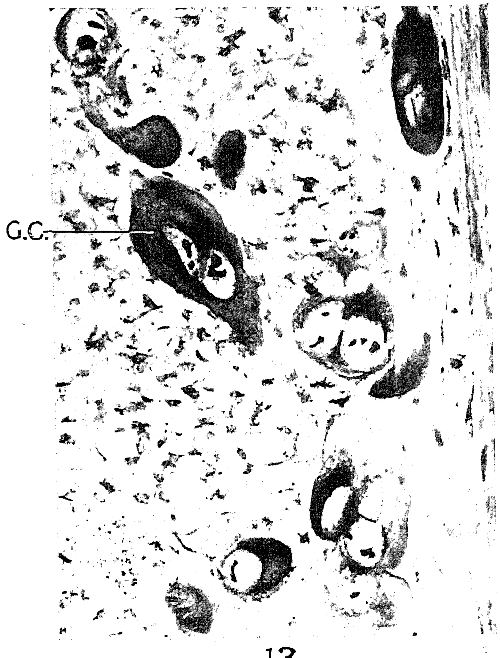

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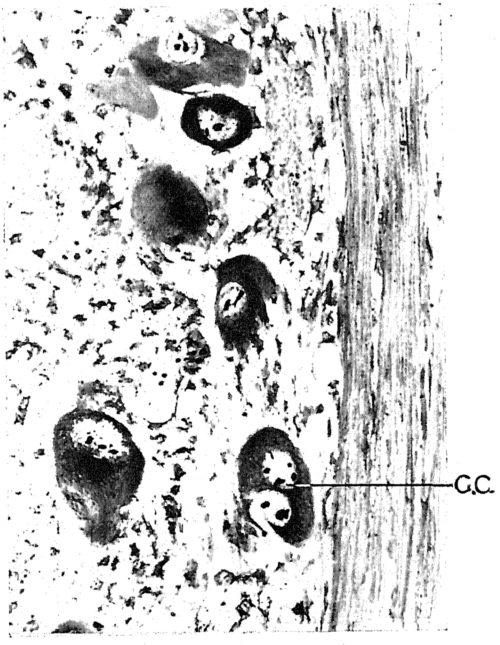

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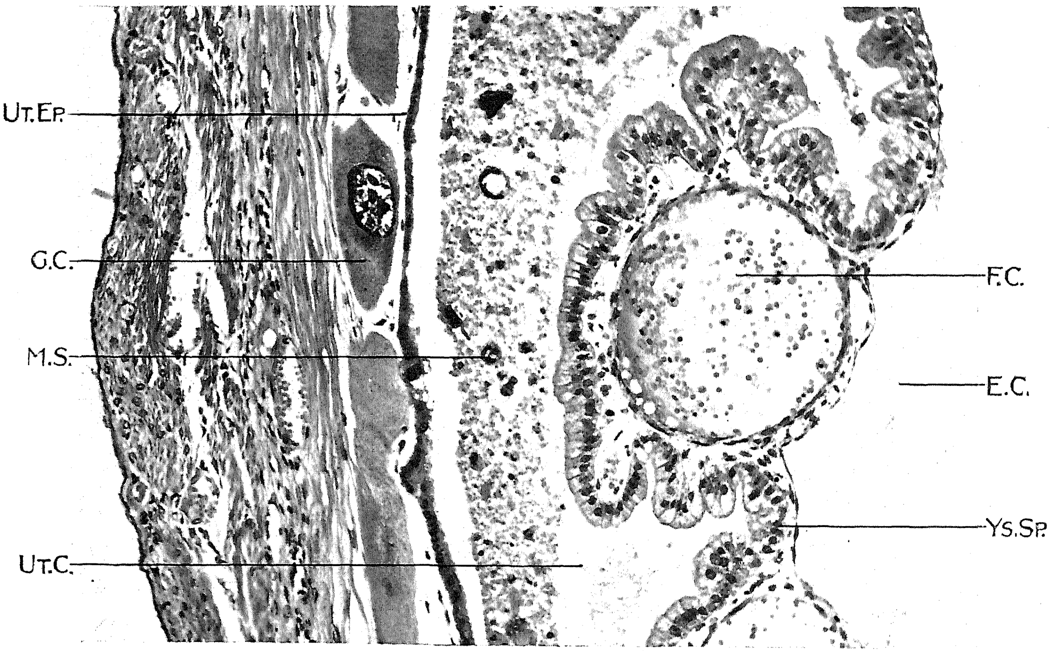

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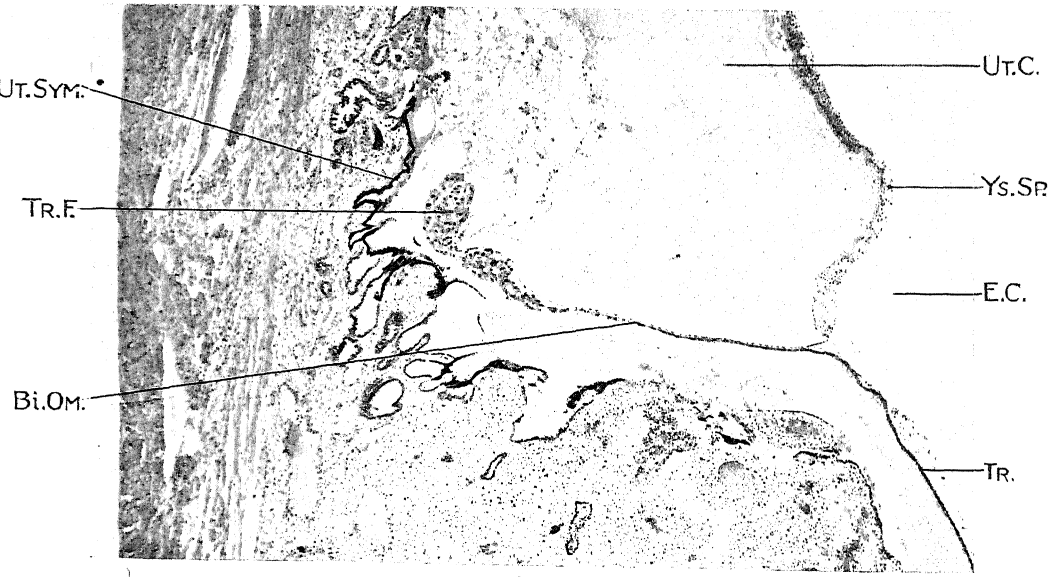

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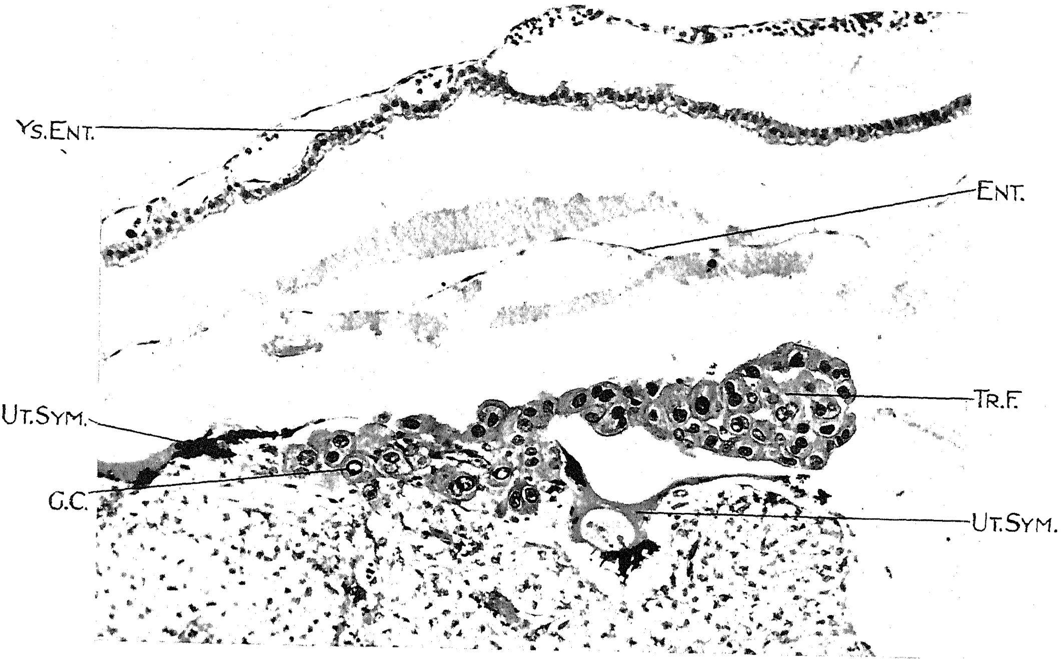

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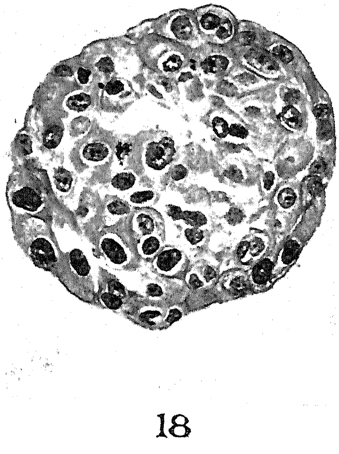

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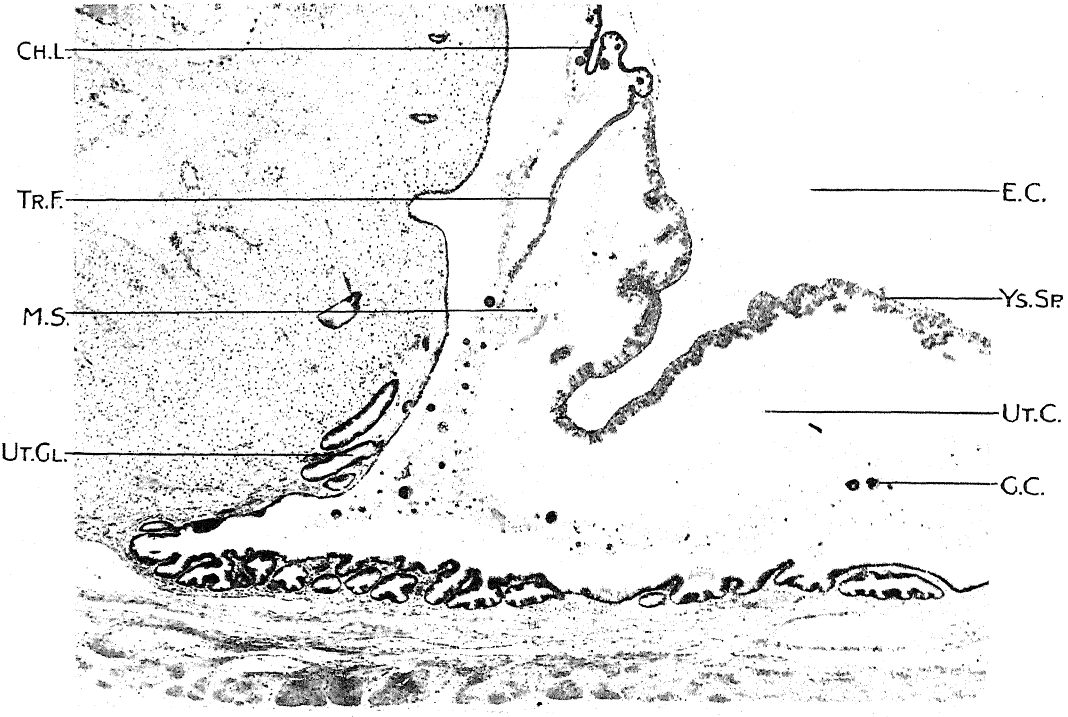

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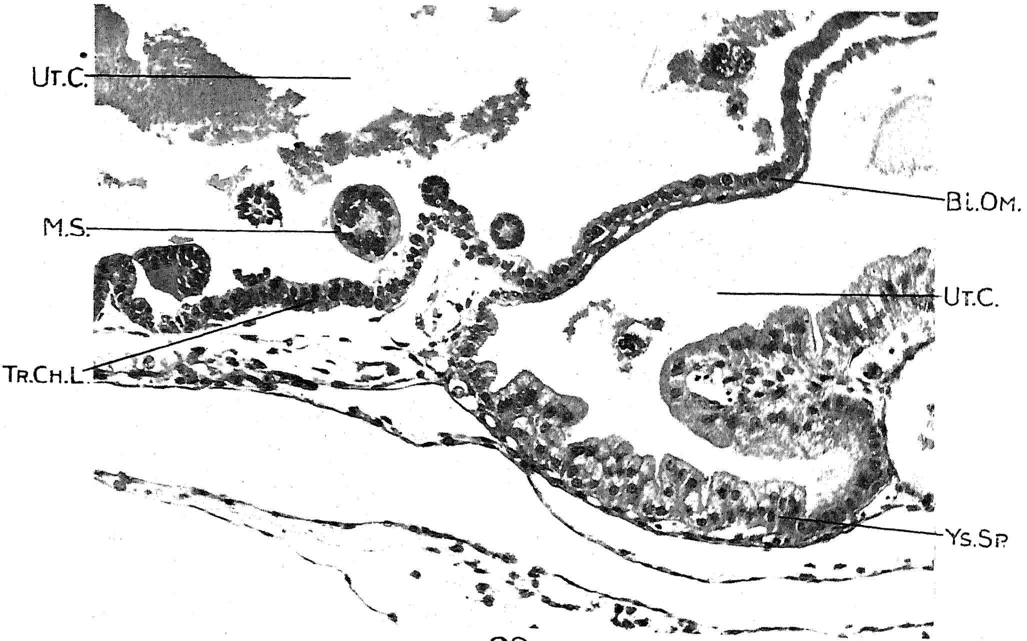

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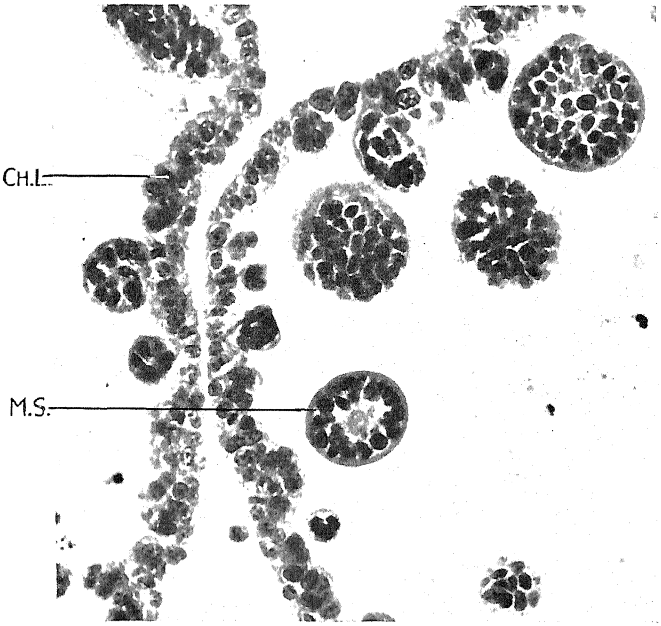

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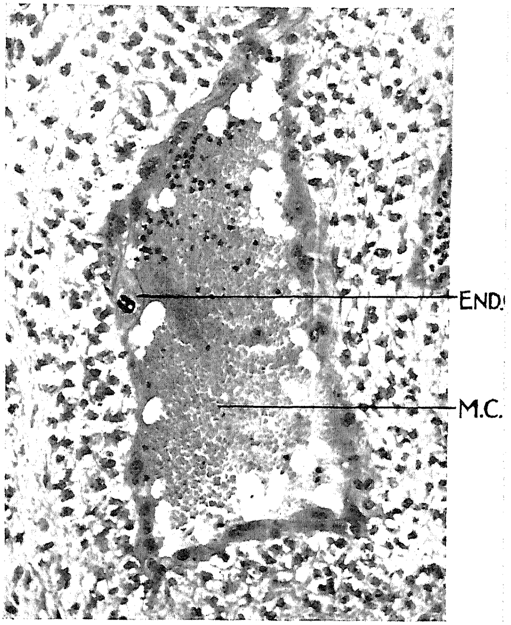

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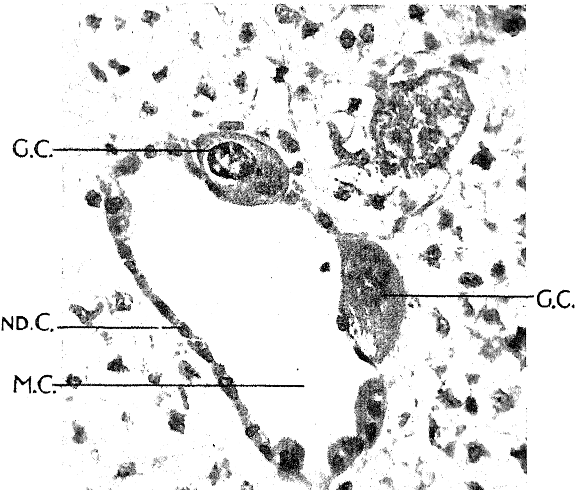

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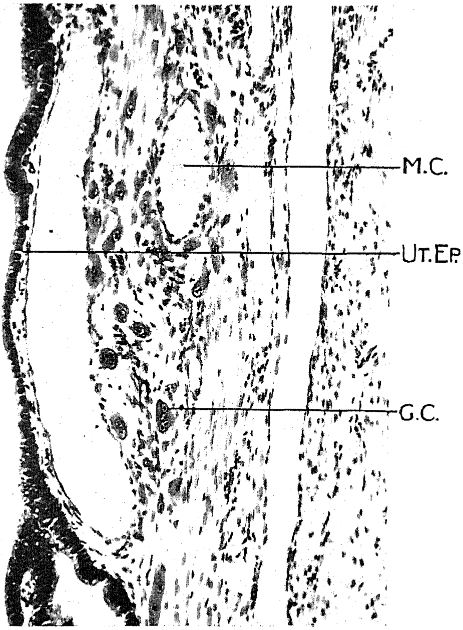

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The Development of Chinese Leishmania *in* Phlebotomus Major *var.* Chinensis *and* P. Sergenti *var.*

By W. S. Patton and Edward Hindle, Members of the Kala Azar Commission of the Royal Society.

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Although it is now more than twenty years since Sergent (1905) suggested the possibility of *Phlebotomus* being responsible for the transmission of Oriental Sore, it was not until 1921 that any direct evidence was produced in support of this hypothesis. In that year Sergent, Parrott, Donatien and Beguet (1921) collected a large number of *P. papatasii* from a neighbourhood infected with Oriental Sore and inoculated their contents into human subjects; subsequently, the characteristic lesions developed at the sites of inoculation, from which typical *Leishmania* were recovered. Similarly, Aragao (1922) produced in a dog a cutaneous nodule containing *Leishmania*, by inoculating crushed sandflies (*P. intermedius*) that had been fed on a case of Brazilian Leishmaniasis 13 days previously.

The first demonstration of the development of *Leishmania* in the sandfly, however, was furnished by Knowles, Napier and Smith (1924), who found flagellates in the gut of *Phlebotomus argentipes* fed on Kala Azar patients in Calcutta. This discovery has been confirmed and extended by the subsequent investigations of the Indian Kala Azar Commission. Christophers, Shortt and Barraud (1925, A and B), working in Assam, also found that *L. donovani* developed in the midgut of *P. argentipes* fed on suitable cases; the infection was especially well developed on the fifth day, the flagellates being found attached to the wall of the gut up to the hinder end of the pharynx. Finally, Shortt, Barraud and Craighead (1926) carried the development a stage further by demonstrating the presence of flagellates in the buccal cavity, in close proximity to the commencement of the biting parts. These results, combined with the careful epidemiological investigations recorded in Report No. 1 of the Indian Kala Azar Commission (1926), would seem to justify the view held by this Commission that the sandfly is probably the vector of the parasite of Kala Azar. Moreover, the recent observations of Adler and Theodor (1926), who succeeded in producing infection with Oriental Sore by the inoculation of flagellates from the alimentary canal of *P. papatasii* previously fed on an infected case, shows that in this closely related species of *Leishmania*, the sandfly is certainly capable of harbouring the organism in an infective form.

The existence of an extensive area of endemic Kala Azar in North China offers a good opportunity of studying the transmission of this variety of the disease, and in recent years the China Medical Board of the Rockefeller Foundation has supported an investigation into the problem. Young and Hertig (1926), who have been in charge of this inquiry, have recently described the development of flagellates in sandflies fed on hamsters infected with the local strain of Kala Azar. Two species of *Phlebotomus* were used in their feeding experiments, *Phlebotomus major* var. *chinensis*, and a species referred to by them as *Phlebotomus* " C," which we have been able to identify as the Mesopotamian variety of *P. sergenti*.

They obtained entirely negative results with both species when fed on human Kala Azar patients, but by using infected hamsters 29 out of 34 *P. major* showed development of flagellates in the alimentary canal, whilst under similar conditions only 7 out of 233 *Phlebotomus* " C " became infected. These results differ considerably from those obtained with *P. argentipes* in India, but in spite of the negative results of the feeding experiments with human cases, the ready development of flagellates in *P. major* fed on infected hamsters suggests the possibility of this insect being a carrier.

METHODS.

The sandfly season in China is extremely short, an indication of this being the popular idea that sandflies arrive at the time of wheat harvest (the end of May) and disappear early in July, soon after the rainy season starts and mosquitoes become numerous. As far as our experience goes, this is certainly true in the case of *P. major* var. *chinensis* which, normally, seems to have only one brood a year; but both *P. sergenti* var. and *P. perturbans* var. have a longer duration and occur from the end of May to the end of September, although very few individuals can be found in the last few weeks of the season. In China there are many difficulties in the way of collecting sandflies, as, for obvious reasons, it is almost impossible for a European to enter the houses of the inhabitants and search in the localities where they are most prevalent. A few were obtained in the hospital buildings, dormitories, etc., and also in some of the mission compounds and temples; but this supply was quite inadequate for any experimental work, and it was necessary to devise some way of obtaining larger numbers of these insects.

Through the help of our assistant, Mr. Feng Lan Chow, collecting tubes were distributed among the coolies of the Hospital and University, and a few coppers offered for each live sandfly brought to the laboratory. The news soon spread round the neighbourhood and for some weeks large numbers of sandflies were brought in by men, women and children. In Tsinan, the flies were all either *P. major* var. *chinensis*, or *P. sergenti* var., and it is interesting to note that, with few exceptions, practically no other insects were mistaken for sandflies, and after being shown the difference even small children were able to distinguish between males and females. This was in marked contrast to the responses to our numerous requests for sandflies made to friends in various parts of China, who rarely seemed to be able to distinguish between *Phlebotomus* and other small insects, both biting and non-biting, a point to be remembered in view of the almost universal statement that sandflies were common in their respective localities.

The flies were brought to the laboratory in every conceivable receptacle, ranging from large broken lamp chimneys patched with paper down to miniature scent bottles and vaccine capsules. Sometimes as many as 20 flies were brought in a single test-tube, and any one who has had experience in handling these insects will realise the skill and patience required to collect so many in such a receptacle. At the beginning of the season we paid about threepence for each fly, but this price was soon reduced to about a halfpenny for a female and a farthing for a male. In 1926 the first sandfly was caught on 22nd May, and during the following week the numbers gradually increased until early in June, when about 100 flies were being brought in daily. For the time being a brisk trade arose in the neighbourhood of the Hospital (with even middlemen !) and the numbers brought to the laboratory rapidly increased until about the middle of June, when on one occasion as many as 1,500 flies were collected in a day, but after the end of June there was a very rapid diminution, especially in the case of *P. major*. *P. sergenti* var. was always much more abundant than *P. major*, and after the middle of July only odd specimens of the latter species were obtained. *P. sergenti* var , on the other hand, although in diminished numbers, continued to be brought to the laboratory up to the end of September.

Our method of handling the sandflies is a modification of that described by Young and Hertig (1926), and was very kindly demonstrated to one of us by Dr. Hertig himself. On being brought to the laboratory the flies were counted (only for purposes of payment) and then liberated into a rectangular cage made of silk bolting cloth (No. 40 mesh) and provided with a sleeve. The details of this cage, which possesses the advantage of extreme simplicity, are shown in the accompanying fig. 1.

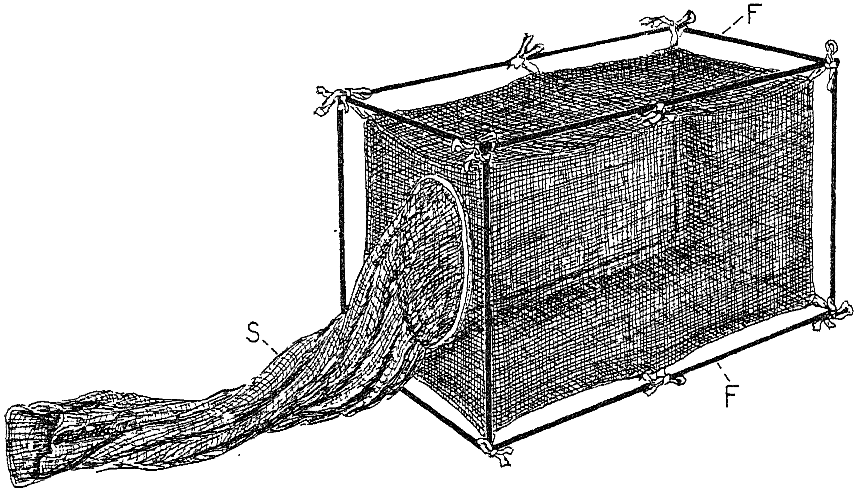

Fig. 1.—Cage used for sorting out the sandflies and transferring them from one receptacle to another. F, strong wire frame to which the cage is fastened by means of tape; if desired this frame can be made collapsible. S, sleeve conveniently wide enough to admit the arm. Any joinings in the cage, such as both ends and the attachment of the sleeve, should be strengthened by means of tape.

The insects were not kept in the cage longer than necessary, as under such conditions they die within a comparatively short time, though if necessary they may be kept alive for a longer period by covering the cage with a damp cloth. As soon as possible the flies were transferred either to earthenware pots or to feeding boxes, the transference being effected by the use of a "fly pipette" constructed as follows:—

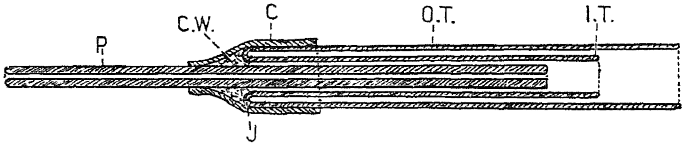

Fig. 2.—Diagram showing construction of "fly pipette." C, indiarubber tubing. CW, cotton-wool packing between indiarubber tubing and plunger. IT, inner tube, and OT, outer tube joined together by sealing-wax at J. P, plunger.

A piece of glass tubing 10 cm. in length with an internal diameter of about 0·5 cm. is fitted inside a wider glass tube about 11 cm. in length. These two tubes are fixed together at one end by means of sealing wax, and consequently at the opposite end the larger tube overlaps the inner one by about 1 cm. A plunger is now made by cutting off a piece of capillary glass tubing about 12 cm. in length, which fits exactly into the inner tube ; it is kept in position by a short piece of india-rubber tube fitting tightly over the joined ends of the glass tubes, and loosely around the plunger ; this space between the two is packed with cotton-wool sufficiently tightly to hold the plunger in position, but not to interfere with its free movement up and down the inner tube. Details of this pipette are shown in the accompanying diagram (fig. 2).

The flies are caught in the open end of the pipette which is then closed by the forefinger. In order to facilitate examination with a lens, the plunger is pushed forward until it is level with the free end of the inner tube, when the fly will be confined to the small space at the end of the pipette. After examination the plunger is drawn back and the fly transferred either to a breeding pot or to a feeding box (described below), both of which are fitted with glass entrance tubes of approximately the same diameter as the inner tube of the fly pipette. The outer tube of the pipette fits over this entrance tube and the fly can then be blown into the receptacle by a gentle puff of air down the capillary tube forming the plunger. It is essential that all parts of this pipette should be kept as clean and dry as possible, as the sandflies readily adhere to any moist surface.

Young and Hertig used a similar device, but with a solid plunger, so that the flies had to be pushed into the receptacles instead of being blown into them. This process has to be done with care, otherwise the fly is liable to be caught between the plunger and the tube. By employing a capillary tube as plunger this risk is avoided and the flies can be transferred much more quickly.

The pots used for breeding sandflies, and also for feeding large numbers of them, were prepared from unglazed earthenware jars, resembling small honey pots, about 7·5 cm. high and 8 cm. in diameter, with a well marked lip and a flat base (fig. 3). In one side of the pot a hole is drilled, through which a piece of glass tubing about 5 cm. long is inserted and fixed in position by means of sealing wax, in such a manner that half the tube projects inside the pot and half outside. This tube must be of about the same bore as the inner tube of the fly pipette in order to allow the introduction of flies as described above. The pot is then lined with a thin layer of plaster of Paris in order to facilitate subsequent examination. The mouth of the pot is closed by a piece of bolting cloth held in position by a rubber band. When in use the pot is kept in a saucer containing wet cotton-wool in order to keep the inner surface moist, but not wet, as in this case the flies are liable to adhere to it and thus get killed.

The feeding boxes (figs. 4 and 5) are a modification of those commonly used for feeding lice (*vide* Nuttall, 1917), and were prepared by replacing the bottom of a small cardboard pill-box with a layer of bolting cloth about No. 40 mesh.

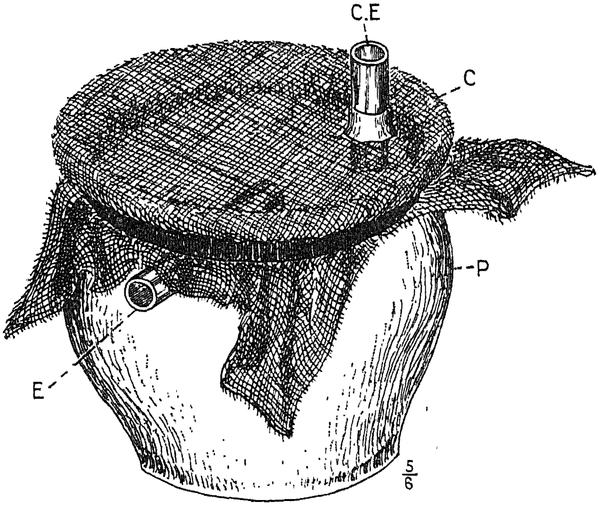

Fig. 3.—Breeding pot. P, earthenware pot lined with plaster of Paris. E, entrance tube through wall of pot. C, piece of bolting cloth forming a cover to the pot, held in position by an indiarubber band. CE, additional entrance tube through cover (sometimes omitted) held in position by adhesive tape at each side of cloth; by inserting a piece of straight wire, moistened at the tip, through this tube, a dead fly can be removed without opening the cover and having the labour of re-catching any remaining live flies.

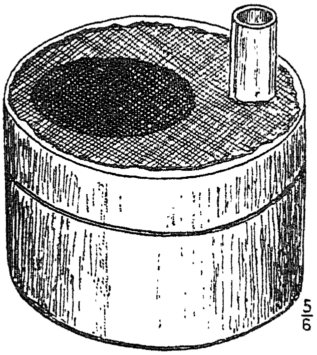

Fig. 4.—Feeding box, showing position of entrance tube and "window" in lid of pill box.

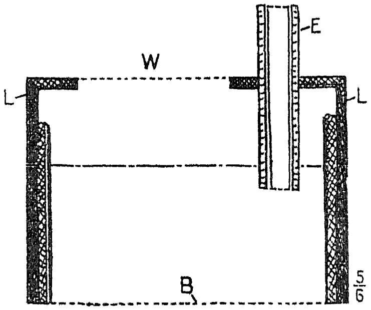

Fig. 5.—Diagrammatic section of feeding box. E, entrance tube. B, bolting cloth through which insects feed. L, lid of pill box. W, window, made of bolting cloth.

A small window was then made in the lid of the pill-box by cutting out a circular piece and gumming a piece of bolting cloth over the aperture. A short piece of glass tube is fixed in the lid of the pill-box to serve as an entrance tube for the introduction of sandflies by means of the pipette. It is necessary to keep this entrance tube plugged with cotton-wool after the flies have been introduced, as in such a confined space they are liable to discover the way out.

Feeding.

When large numbers of flies were available for feeding experiments, they were introduced into the pots and the bolting cloth applied to the skin of the subject. If hungry the insects may start feeding at once, and in any case if they have not fed within half-an-hour it can safely be assumed that the flies do not want to feed. As a general rule they only feed well at night time, though if very hungry they will bite during the day. When the insects are feeding through the bolting cloth it is necessary to avoid any lateral movements of the receptacle, as if this happens the mouth parts are liable to be injured by the threads of the cloth. Although males were generally introduced together with females we were unable to find that they in any way affected the feeding, for pots containing only virgin females fed quite as well as those containing both sexes.

When dealing with smaller numbers the flies were introduced into the special feeding boxes described above, and then fed either on patients or on hamsters. When feeding on a hamster its belly was first shaved, the animal then tied down on its back, and the feeding box containing the insects held against the shaved surface for about half an hour. In dealing with the Giant Hamster (*Cricetulus triton*) it was found advisable to narcotise the animal previously in order to keep it quiet. Subcutaneous injections of Luminol were found to be very satisfactory, and in spite of repeated inoculations at short intervals we have never observed any ill-effects from its use. Apart from the fact that a narcotised animal is much easier to handle, the sandflies feed better, as they are not disturbed by any sudden movements of the abdomen.

Examination of the flies.—After having had an opportunity of feeding, the flies were liberated in a large cage and caught and examined individually in order to see whether they had fed or not. In the case of sandflies whose gut was previously empty the presence of blood was readily observed, as the red colour is preserved for at least 24 hours. When undigested black blood was present in the gut, it was sometimes not easy to distinguish the presence of a small quantity of red blood, but as a rule there was not much difficulty in deciding which flies had fed recently. The fed flies were introduced into the earthenware pots described above, and kept either at room temperature (about 30° C.) or in a cool incubator at 20° C. Any which died were at once removed and dissected in order to follow the development of the parasite.

For dissection the fly is placed in a test-tube containing a little (0·7 per cent.) saline, and the tube shaken in order to wet the fly which, if alive, is thus rendered helpless. It is then placed in a drop of saline on a slide and the wings and legs removed; the body is then transferred to another drop of saline in order to get rid of the hairs and loose appendages. By means of two very fine mounted needles* the head is now loosened from the thorax, the neck being severed without breaking the alimentary canal. In order to do this one needle is placed in the thorax and the other across the head; then by gentle traction the cervical membrane can be broken and the head, with the alimentary canal still attached, freed from the thorax. This process also exposes the salivary glands which are drawn out of the thorax, as they remain attached to the salivary duct. At this stage it is possible to examine the front part of the alimentary canal for the presence of flagellates, which can be detected without much difficulty through the transparent walls of the gut. Next the dorsal part of the thorax containing the muscles is cut off by a stroke of the needle, thus liberating the anterior parts of the midgut. The only difficult operation is to free the anterior end of the midgut from the chitinous ring of the front part of the prothorax, which is effected by careful manipulation with the two needles.

The remaining portion of the thorax, including the bases of the legs, can easily be removed, but it is important to remember that the anterior end of the alimentary tract is situated in the central part of the thorax just above the bases of the legs. The last segment of the abdomen is now cut off in order to liberate the hinder end of the alimentary canal. If there is not too much blood in the stomach the whole alimentary tract can now be drawn out of the abdomen by gently pulling on the head, at the same time holding the abdomen by a needle through the first segment. If there is too much blood it is advisable to cut open the anterior abdominal segments in order to allow the exit of the distended stomach. The presence of flagellates can readily be observed in all parts of the alimentary canal, except in the case of the stomach when it contains much undigested blood. The walls of the anterior and posterior parts of the gut are extremely transparent, and it is possible to make whole preparations to show the flagellates *in situ*. The specimen is transferred into a drop of saline on a cover-glass and the various parts of the alimentary tract are arranged in position; excess saline is then removed by means of filter paper. When the preparation is just about to dry it is fixed to the cover-glass by flooding it with a drop of Bles' fluid; it can then be stained and mounted in the usual way. As a general rule, however, the contents of an infected gut were teased up in saline and either inoculated into hamsters or, if stained preparations of the flagellates were desired, smears were made in the usual way.

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* We are much indebeted to Lt.-Col. Christophers, I.M.S., who very kindly supplied us with these needles, of No. 16 gauge, a size which has to be specially ordered, as it is no longer on the market. The mounted needles were prepared by forcing the base of the needle into a soft wooden handle until only about 1 cm. was left free.

RESULTS OF FEEDING SANDFLIES ON HUMAN CASES OF CHINESE KALA AZAR.

A.—*Experiments with Phlebotomus major* var. *chinensis.*

In view of the large percentage of *Phlebotomus argentipes* showing development of flagellates after being fed on cases of Indian Kala Azar, we were inclined to the assumption that it would not be difficult to obtain evidence of a similar development in sandflies fed on patients infected with Chinese Kala Azar. However, Young and Hertig (1926) in their experiments obtained entirely negative results when *P. major* were fed on human cases, and at Tsinan we have only twice been able to obtain any positive results.

In all these experiments sandflies caught in nature were used and consequently the great majority of them had already fed previously. The flies were fed on seven untreated cases of Kala Azar, of which all except one were of long duration, with greatly enlarged spleen and other well marked symptoms of the disease. A total of 102 *P. major* were fed on these patients and subsequently examined at intervals ranging from two to eight days without any of the insects being re-fed. Development of flagellates was observed only in two experiments as follows:—

(I) *Case No.* F1702.—Woman, aged 39; first noticed symptoms about two months previously; liver and spleen only slightly enlarged. Twelve *P. major* were fed on this patient and subsequently kept at about 30° C. After three days two were dissected, of which one contained many flagellates in the midgut, whilst the other was negative. Six days after feeding another five were examined, one contained a few flagellates in the midgut, the other four were negative. The remaining five were killed the following day; two were heavily infected in the anterior part of the midgut, the other three were negative. In this experiment, therefore, four out of twelve sandflies became infected. Unfortunately the patient left the hospital the next day and no further examination of the case was possible. It may be noted that this was by far the mildest case of Kala Azar used in any of our feeding experiments.

(2) *Case No.* F1667.—Boy, age 15 years, with greatly enlarged spleen and history of 18 months' duration of symptoms.

In all, 53 *P. major* were fed on this patient on four successive days and subsequently examined after intervals ranging from three to six days. One sandfly showed a very scanty infection of the midgut four days after being fed, whilst the remaining 52 flies were all negative.

Summarising the results of our experiments with *P. major*, it will be seen that out of 102 flies fed on human patients infected with Kala Azar, all diagnosed by liver puncture, only five insects showed any subsequent development of flagellates.

B.—*Experiments with Phlebotomus sergenti* var.

A total of 202 sandflies belonging to this species were fed on eight untreated Kala Azar patients, and examined after intervals of two to ten days without any of the insects being re-fed. In no case was any development of flagellates observed, but as a number of these flies were dissected more than three days after the feed it is possible that some infections may have died out, especially in view of the results of our experiments with hamsters recorded below.

RESULTS OF FEEDING SANDFLIES ON HAMSTERS INFECTED WITH CHINESE KALA AZAR.

A.—*Experiments with Phlebotomus major* var. *chinensis*.

The hamsters used in the following experiments had all shown parasites in the peripheral blood, as determined by the examination of stained films. Accordingly, they must be regarded as selected cases, for employing this method parasites were only found in the blood of less than 20 per cent. of the infected hamsters. Whether this selection may have affected our results or not is uncertain, in view of the fact that some of our most successful feeding experiments were made on a hamster in which no parasites could be found in films made at the time of the experiment, although this animal had shown parasites in its blood on a previous occasion. In the majority of these experiments sandflies caught in nature were used, and as such flies presumably had fed at least once before being brought to the laboratory, they were starved for two or three days before being fed on an infected hamster. After having been fed, the flies were kept in breeding pots at either the room temperature, approximately 30° C., or in a cool incubator at 20° C. They seemed to live equally well at both temperatures, but as one would expect the development of flagellates and also the digestive processes took place more rapidly at the higher temperature. As a general rule the flies were removed for examination only after they had died, as we wished to leave as long an interval as possible for any subsequent development of the parasites. Very few of these flies were re-fed as it was decided that it would be more satisfactory to employ laboratory bred flies for any transmission experiments. Unfortunately this species seems to have only one brood a year, and the few individuals that completed their development during the summer were very difficult to feed and rarely fed more than once.

The results of our experiments are given in the following table :—

Date when fed.	No. of flies fed.	Bred or Caught.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
31.5.26	3	Caught	30	81	6	1	Midgut with heavy flagellate infection extending to proventriculus.
					7	1	As above, but in addition numerous flagellates in intestine.
					8	1	Negative.
1.6.26	3	Caught	30	80	2	1	Negative.
					7	1	Numerous flagellates in midgut and intestine.
					8	1	Positive.
9.6.26	33	Caught	30	81	1	1	Negative.
					3	1	Developmental forms in midgut.
					4	1	Many flagellates in midgut.
					6	14	All heavily infected in midgut.
10.6.26	10	Caught	30	81	6	5	All positive in midgut.
					7	5	Three positive in midgut ; two negative.
10.6.26	2	Caught	30	112	5	1	Negative.
					6	1	Very heavy infection of midgut.
11.6.26	2	Caught	30	90	5	2	Both infected in midgut.
12.6.26	21	Caught	30	90	5	9	All positive in midgut.
13.6.26	30	Caught	30	109	3	2	Both negative.
					5	3	Two negative. One contained very few flagellates in midgut.
					6	10	Four negative and six positive. Infection confined to midgut and scanty in all except one.
					9	1	This fly was re-fed 3 days previously. Very heavily infected ; flagellates in pharynx, œsophagus, midgut and hindgut. The fæces also contained flagellates.
14.6.26	45	Caught	30	90	2	1	Positive in midgut.
					5	2	Both positive in midgut.
					6	8	All positive in midgut.
					7	3	All positive in midgut.
					8	11	Nine positive ; two negative.
					9	1	Positive.

Date when fed.	No. of flies fed.	Bred or caught.	Temperature. °Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
22.6.26	15	Caught	20	81	4	1	Negative.
					5	1	Heavily infected in midgut.
					5	2	One heavily infected in midgut, other degenerate.
					7	2	These flies were both re-fed on a normal hamster the previous day. Both were heavily infected with flagellates in the midgut.
23.6.26	7	Caught	20	81	2	4	Three negative ; one positive.
					4	1	Negative (? degenerate).
					6	1	Heavily infected in midgut.
					10	1	Heavily infected in midgut.
25.6.26	13	Caught	20	80	2	2	Both negative.
					4	1	Negative.
					6	2	One negative ; other positive in midgut.
					8	2	Both heavily infected.
					10	1	Positive in midgut.
6.7.26	6	Caught	20	80	3	1	Developmental forms in midgut.
					4	2	Both heavily infected in midgut.
					5	1	Positive in midgut.
					6	1	Positive in midgut.
9.8.26	5	Bred	30	80	4	1	Positive in midgut.
					5	1	Positive.
					6	2	One heavily infected in midgut ; the other negative.
9.8.26	3	Bred	30	81	4	1	Heavily infected in midgut and hindgut.
					5	1	Positive in midgut.
					8	1	Fly dried up.
12.8.26	7	Bred	30	80	3	1	Heavily infected in midgut.
					5	3	All heavily infected in midgut. One of these flies was re-fed the previous day.
					6	3	One very heavily infected in midgut up to proventriculus ; other two with swarms of flagellates in midgut and extending forward into the pharynx and buccal cavity.
14.8.26	10	Bred	30	99	2	1	Negative.
					3	1	Heavily infected in midgut.
					4	6	Three slightly infected in midgut ; three negative.
					5	2	One positive in midgut ; one negative.
15.8.26	18	Bred	30	119	3	1	Positive in midgut.
					4	1	Positive in midgut.
					5	2	Both positive in midgut.
					6	7	Two negative ; five positive in midgut.
16.8.26	7	Bred	20	116	3	1	Positive in midgut.
					4	2	Both positive. In one numerous active flagellates in pharynx.
					8	1	Very heavily infected in midgut, œsophagus and pharynx.

Date when fed.	No. of flies fed.	Bred or caught.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
18.8.26	3	Bred	30	212	3	2	One positive in midgut and also in œsophageal diverticulum; one negative.
					5	1	Positive in midgut.
18.8.26	2	Bred	30	252	4	2	Both positive in midgut.

Summarising the results of the above described experiments, it will be seen that out of 157 sandflies dissected, flagellates were observed in 122, while 35 were negative. It is probable, however, that in some of the flies recorded as negative the flagellates may have disappeared, owing to degeneration of the contents of the gut following the death of the insect. It is evident, therefore, that *P. major* is a very favourable host for the development of the parasite, and one is led to assume that the ingestion of living *Leishmania* invariably produces infection of the alimentary tract. In the great majority of the flies the flagellates were confined to the midgut; at first they develop in the broad posterior region, but subsequently move forwards into the narrower anterior region, where they were noted frequently attached to the proventriculus and the lining of the gut. Sometimes the whole of the midgut from one end to the other was lined with flagellates forming an almost continuous layer. Although the parasites developed so readily in the midgut, in only five sandflies have we found flagellates extending forward into the pharynx; in two of these individuals they could also be seen in the buccal cavity in the neighbourhood of the opening of the salivary duct. Of those flies showing infection of the pharynx, the intervals between the infective feed and dissection were respectively four, six (two individuals), eight and nine days. In one instance flagellates were observed in the œsophageal diverticulum. In three cases the infection extended backwards into the intestine, in one of them flagellates being observed also in the fæces.

It will be noticed that the few flies which were re-fed all showed very heavy infections, and it is evident that the parasites in the gut are not adversely affected by the ingestion of fresh blood.

After having been fed, the longest period we have been able to keep this species of sandfly alive is 10 days, but the majority of them died before the expiration of a week.

B.—*Experiments with Phlebotomus sergenti* var.

In Tsinan this is by far the most abundant species of sandfly, and as, in addition, it occurs from the end of May to September, we were enabled to perform a much longer series of experiments than in the case of *P. major*. The sandflies were invariably caught specimens, and in the majority of instances had fed previously, so in view of our results it is evident that this species will feed at least four times, as we had no difficulty in re-feeding such caught flies three times. *P. sergenti* differs markedly from *P. major* in the comparative rapidity with which digestive processes take place. As a general rule after a full meal of blood very little trace of undigested food material could be seen in the midgut after an interval of three days at 20° C., and even after two days many of them could be re-fed, especially if they had been kept at 30° C. On the other hand, undigested blood persisted for at least seven days in the gut of *P. major*, and this species required a correspondingly longer interval before it could be re-fed. The flies were kept in the laboratory and fed in exactly the same way as *P. major*. The results of our experiments are given in the following table :—

No. of experiment and date when fed.	No. of flies fed.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
P 1 10.6.26	22	25	80	3	2	Both positive in midgut.
				6	2	Both positive in midgut.
				9	18	Re-fed three days previously on normal hamster. One positive ; remaining seventeen negative.
P 3 14.6.26	74	25	80	4	6	Two negative ; four positive in midgut.
				5	16	Twelve negative ; four positive.
				7	28	Re-fed two days previously. All negative.
P 4 15,6,26	38	25	80	7	17	All negative.
P 5 16.6.26	21	25	109	4	10	Six positive ; four negative.
				5	8	Two positive ; six negative.
P 6 19.6.26	7	25	81	3	4	Three positive ; one negative.
S 20 20.6.26	64	25	80	2	24	Twelve positive ; twelve negative.
				3	10	Six positive ; four negative.
				4	20	One positive ; nineteen negative.
S 21 21.6.26	65	20	80	2	7	Four positive ; three negative.
				3	12	Six positive ; six negative.
				4	14	Eight positive ; six negative.
				5	6	Three positive ; three negative.
				6	5	All negative.
				7	14	Re-fed two days previously. One positive ; thirteen negative.

No. of experiment and date when fed.	No. of flies fed.	Temperature, °Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
S 23 23.6.26	30	20	81	2	6	Four positive ; two negative.
				3	3	All positive.
				6	6	One positive ; five negative.
				7	2	Re-fed two days previously. One positive ; one negative.
				9	3	Re-fed four days previously. Two heavily infected in midgut ; one negative.
				10	3	Re-fed five days previously. Two positive ; one heavily infected in hindgut as well as midgut. One negative.
				12	3	Re-fed seven days previously. All negative.
S 24A 25.6.26	70	20	99	2	10	Two positive ; eight negative.
				3	8	One positive ; seven negative.
				4	9	Four positive ; five negative.
				5	10	Four positive ; six negative.
				6	10	Six positive ; four negative.
				7	9	One positive ; eight negative.
				10	6	All negative.
S 24B 25.6.26	55	30	99	2	7	Four positive ; three negative.
				3	17	Six negative ; eleven positive. Three with flagellates extending to proventriculus.
				4	9	Seven negative ; two positive with flagellates extending to proventriculus, and in one also present in hindgut.
				5	15	All negative.
S 25 28.6.26	11	30	80	8	4	All negative.
				9	5	Re-fed previous day. All negative.
S 27 16.7.26	30	20	109	3	6	One positive : five negative.
				4	4	One positive ; three negative.
				5	4	All negative.
				7	5	Re-fed two days previously. All negative.
				8	11	Re-fed three days previously. Two positive; nine negative.
S 29 20.7.26	37	30	80	3	12	Two negative ; ten positive. Two with flagellates extending to proventriculus.
				5	4	Re-fed two days previously. Two negative ; two positive.
				6	6	Re-fed three days previously. Four positive ; two negative.
				7	1	Re-fed twice respectively four days and one day previously. Positive.
				9	1	Re-fed twice as above. Negative.
S 30 21.7.26	23	20	80	3	2	Both positive.
				5	2	Re-fed two days previously. Both positive.
				6	1	Re-fed three days previously. Positive.
				7	1	Re-fed twice. Negative.
				9	6	Re-fed six days previously. One positive ; five negative.
				12	1	Re-fed twice. Negative.

No. of experiment and date when fed.	No. of flies fed.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
S 31 23.7.26	40	20	212	3	14	Three positive; eleven negative.
				6	10	Re-fed three days previously. All negative.
				7	8	Re-fed four days previously. One very heavily infected; seven negative.
S 34 27.7.26	45	30	80	2	2	Both positive.
				3	1	Re-fed previous day. Heavily infected.
				4	7	One heavily infected; six negative.
				5	3	All negative.
				6	9	Re-fed four days previously. Two positive with flagellates extending to proventriculus; seven negative.
				7	2	Re-fed twice previously. Both negative.
				8	6	Re-fed twice previously. All negative.
S 36 28.7.26	13	20	80	3	3	One negative. Two re-fed previous day, both positive.
				8	1	Negative. Re-fed twice previously.
				9	2	Both negative. Re-fed twice previously.
				11	3	Re-fed three times. All negative.
S 38 2.8.26	40	30	81	3	6	One positive; five negative.
				4	5	Re-fed previous day. Four heavily infected, of which one with flagellates extending up to proventriculus. One negative.
				5	7	Re-fed two days previously. Five negative; two positive. One of these with flagellates extending to proventriculus.
				6	9	Re-fed three days previously. Five negative; four positive, three of these with infection extending to proventriculus.
				6	1	Re-fed twice, respectively one day and three days previously. Positive.
				8	1	Re-fed twice, respectively two and four days previously. Positive, infection extending to proventriculus.
S 39 3.8.26	40	20	80	2	17	All negative.
				3	8	One negative; seven positive.
				4	8	Re-fed previous day. One negative; seven positive.
				5	2	Re-fed two days previously. Both positive.
S 43 7.8.26	40	20	81	3	7	One negative; six positive, of which one with infection extending up to proventriculus.
				4	5	Re-fed previous day. Four negative; one positive.
				5	4	Re-fed two days previously. Two negative; two positive.
				6	4	Re-fed three days previously. Two negative; two positive.
				7	2	Re-fed twice, respectively two and four days previously. One negative; one positive.
				8	2	Re-fed twice, respectively three and five days previously. One negative; one positive, with infection extending up to proventriculus.
				9	1	Re-fed three times. Negative.

No. of experiment and date when fed.	No. of flies fed.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
S 44 7.8.26	37	20	80	3	9	Seven negative ; two positive.
				5	7	Re-fed two days previously. Two negative ; five positive.
				6	3	Re-fed three days previously. One negative ; two positive.
				7	1	Re-fed four days previously. Negative.
				8	6	Re-fed twice, respectively three and five days previously. Four negative ; two very heavily infected.
				9	1	Re-fed twice. Negative.
S 49 12.8.26	28	30	99	2	2	One negative ; one positive.
				3	7	Five negative ; two positive.
				4	5	Four negative ; one positive.
				4	1	Re-fed previous day. Slight infection of midgut.
				6	6	Re-fed three days previously. All negative.
				7	3	Re-fed twice, respectively two and four days previously. One negative ; two positive.
S 50 12.8.26	30	20	80	2	1	Negative.
				3	4	One negative ; three positive.
				4	2	Both positive.
				6	7	Re-fed three days previously. Five negative ; two positive.
				7	6	Re-fed four days previously. All negative.
S 51 14.8.26	35	30	84	2	7	Four negative.
				3	3	One negative ; two positive.
				3	2	Re-fed previous day. Both negative.
				4	4	Re-fed two days previously. All positive.
				5	3	Re-fed three days previously. Two negative ; one positive.
				6	8	Re-fed four days previously. Seven negative ; one positive.
				7	1	Re-fed twice. Negative.
S 52 14.8.26	28	30	106	2	7	All negative.
				3	12	All negative.
				4	9	All negative. It should be noted that the hamster 106 used in this experiment was heavily infected and had shown parasites in the peripheral blood two days previously.
S 53 14.8.26	13	30	116	2	6	Two negative ; four positive.
				3	4	All positive.
				3	1	Re-fed previous day. Positive.
				4	3	Re-fed two days previously. All negative.
				6	2	Re-fed four days previously. Both negative.
S 58 16.8.26	33	20	119	2	5	All negative.
				3	8	Three negative ; five positive.
				4	5	Four negative ; one positive.
				4	1	Re-fed previous day. Positive.
				5	3	Re-fed two days previously. All positive, one with infection extending to proventriculus.

No. of experiment and date when fed.	No. of flies fed.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
S 58 16.8.26	33	20	119	6	4	Re-fed three days previously. One negative; three positive.
				6	3	Re-fed twice, respectively one and three days previously. Two negative; one positive.
				7	1	Re-fed twice. Negative.
S 59 16.8.26	23	20	212	2	5	All positive.
				3	2	Both positive.
				4	6	All positive. Heavy infection.
				5	3	Re-fed two days previously. All heavily infected.
				6	3	Re-fed three days previously. One negative; two positive, one with flagellates extending up to proventriculus.
				6	1	Re-fed twice, respectively one and three days previously. Heavily infected.
S 60 16.8.26	19	20	221	2	5	All positive.
				3	5	One negative; four positive.
				4	4	One negative; three positive.
				4	2	Re-fed previous day. One negative; one positive.
				5	4	Re-fed two days previously. Two negative; two positive.
S 62 17.8.26	32	30	111	2	6	All negative.
				3	8	Seven negative; one positive.
				5	14	All negative.
S 63 17.8.26	35	30	225	2	11	Six negative; five positive.
				3	6	Three negative; three positive.
				4	4	Three negative; one positive.
				4	1	Re-fed previous day. Negative.
				5	5	Re-fed two days previously. Four negative; one positive.
S 66 18.8.26	31	30	252	3	2	Both positive.
				4	4	Three negative; one positive.
				5	9	All negative
S 67 18.8.26	26	30	221	3	2	One negative; one positive.
				4	4	Two negative; two positive. One of these heavily infected up to proventriculus, although no trace of blood present.
				5	8	All negative.
S 68 19.8.26	22	30	260	2	11	All negative.
				3	11	All negative.
S 69 19.8.26	39	20	116	3	13	Three negative; ten positive.
				4	4	All positive.
				5	4	Re-fed previous day. Two negative; two positive.
				6	8	Re-fed two days previously. One negative; seven positive.

No. of experiment and date when fed.	No. of flies fed.	Temperature, ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
S 71 21.8.26	60	20	212	2	4	One negative; three positive.
				3	2	Both positive. One with heavy infection extending up to proventriculus.
				4	4	One negative; three positive.
				5	2	Re-fed two days previously. Both very heavily infected, one with flagellates extending up to proventriculus.
				6	4	Re-fed three days previously. Two negative; two positive.
				7	1	Re-fed twice; respectively two and four days previously. Heavily infected.
				10	3	Re-fed twice, respectively five and seven days previously. Two negative; one positive.
S 73 23.8.26	118	20	252	2	13	Ten negative; three positive.
				3	27	Two negative; twenty-five positive.
				4	14	Five negative; nine positive.
				5	15	Eight negative; seven positive.
				6	8	Re-fed two days previously. Five negative; three positive.
				8	8	Re-fed four days previously. Four negative; four positive.
S 74 24.8.26	53	30	252	2	7	One negative; six positive.
				3	5	Four negative; one positive.
				4	2	Both negative.
				4	5	Re-fed on previous day. Four negative; one positive.
				5	5	Re-fed two days previously. All negative.
				6	4	Re-fed three days previously. Three negative; one positive.
				7	5	Re-fed twice previously. All negative.
				8	6	Re-fed twice previously. All negative.
				9	3	Re-fed three times. All negative.
S 75 26.8.26	75	30	212	2	6	Five negative; one positive.
				3	8	Two negative; six positive.
				4	12	Seven negative; five very heavily infected, in one case infection extending to proventriculus.
				4	6	Re-fed on previous day. Five negative; one positive.
				5	3	Re-fed two days previously. Two negative; one positive.
				6	4	Re-fed three days previously. One negative; three heavily infected.
				7	4	Re-fed twice, respectively two and four days previously. Three negative; one positive.
				8	5	Re-fed twice. All negative.
				9	3	Re-fed twice. All negative.
				9	1	Re-fed three times, respectively one, four and six days previously. Heavily infected.
				10	1	Re-fed three times. Negative.
S 81 10.9.26	31	20	221	4	5	Three negative; two positive.
				6	9	Re-fed three days previously. Six negative; three positive.

No. of experiment and date when fed.	No. of flies fed.	Temperature. ° Centigrade.	No. of hamster on which flies fed.	No. of days between feeding and dissection.	No. of flies dissected.	Results of dissection and remarks.
S 82 13.9.26	16	20	212	3	4	Three negative ; one positive.
				5	5	Two negative ; three positive.
				7	4	Three negative ; one positive, infection extending up to proventriculus.

Summarising the results of these experiments with *P. sergenti* var. it will be seen that 1,170 sandflies were dissected at intervals ranging from one to twelve days after feeding on infected hamsters. Flagellates were observed in 430, giving an infection rate of about 36 per cent. It should be noticed, however, that in the majority of cases fresh examination was the only method used to decide whether the gut contents were positive or negative, and consequently young developmental forms might easily have been overlooked, which, for example, must certainly have been the case in experiment S 73. In addition, some of the infections may have disappeared owing to degeneration of the parasites following the death of the sandfly, and therefore the true infection rate is probably much higher than recorded in our experiments.

Except where otherwise stated, the flagellates were restricted to the broad posterior region of the midgut. Unlike *P. major* parasites were never found in the œsophagus or pharynx, and in only 23 out of 430 positive flies were they observed in the narrow anterior region of the midgut behind the proventriculus. In two individuals they were present in the hindgut.

As a general rule the flagellates seem to persist only so long as food material remains in the alimentary canal. This is well shown in experiment S 24B, in which the number of infected flies falls from eleven positive and six negative on the third day, to two positive and seven negative on the fourth day, and to fifteen negative (all examined) on the fifth day.

When the flies are kept at 30° C. the development of flagellates seems to attain its maximum about three days after an infective meal, and unless the insect is re-fed the parasites disappear by the fifth day. At 20° C. the maximum development seems to be reached about the fourth or fifth day, after which the flagellates gradually die out unless the insect is re-fed. When the flies are re-fed, however, the infection may persist indefinitely, and flagellates have been found in the midgut up to the tenth day after an infective meal. It will be noticed that the proportion of sandflies becoming infected varies from completely negative, as for example in experiments S 52 and S 68, up to nearly all positive, as in experiment S 59, in which only one fly was negative out of twenty. The significance of these variations has not been fully elucidated, as all the hamsters used in our experiments had shown parasites in the peripheral blood as determined by direct examination. Although the number of parasites circulating in the blood may vary from day to day, it is doubtful whether this fact affords a complete explanation of such widely different results as those recorded above.

The difference between our results and those obtained by Young and Hertig (1926), who record an infection rate of only 3·3 per cent. in the case of *P. sergenti* fed on infected hamsters, can probably be explained by the fact that in their experiments most of the flies were dissected at too long an interval after the infective meal and consequently many of the infections would have died out.

CONCLUSIONS.

The results of the foregoing experiments show that *Phlebotomus major* var. *chinensis* is a very favourable host for the development of the *Leishmania* of Chinese Kala Azar, the results of the feeding experiments with infected hamsters recalling those obtained in India with *P. argentipes* fed on human cases of the disease. The negative results of our feeding experiments with human patients can easily be explained in view of the extreme rarity of the parasites in the peripheral blood. When a fly is infected the flagellates attach themselves to the lining of the midgut and grow forward, under favourable conditions passing into the pharynx and buccal cavity after an interval of about six days. Once infected this species of sandfly seems to remain so for the duration of its life (10 to 12 days), irrespective of whether it is re-fed or not. In view, therefore, of the readiness with which *Leishmania* develops and grows forward in this insect, the presence of flagellates in the neighbourhood of the biting parts of infected flies, and the ease with which they may be re-fed, there is strong presumptive evidence in support of the view that *P. major* var. *chinensis* is concerned in the transmission of the parasite of Kala Azar in North China.

The results obtained with *P. sergenti* var. were of quite a different character. There was no difficulty in obtaining development of flagellates in the midgut of this species fed on infected hamsters, but, possibly as a result of the more rapid digestive processes, the parasites as a general rule died out after three or four days, unless the flies were re-fed. Under these circumstances the flagellates could be kept alive during the life-time of the insect, but the type of development was very dissimilar from that in *P. major*. Instead of the flagellates becoming attached to the wall of the midgut and growing forward into the anterior part of the alimentary canal, the infection was generally restricted to the broad posterior region of the midgut, where the parasites occurred amongst the remains of undigested food material. In no case were any flagellates found invading the œsophagus, although 430 infected flies were dissected. It is evident, therefore, that *P. sergenti* var. is a much less favourable host than *P. major* var. *chinensis*. On the other hand, the readiness with which flagellates develop in *P. sergenti*, and the fact that they can be maintained in the gut by re-feeding, which doubtless occurs in nature, suggests that this species cannot be entirely ignored in considering any possible vectors of the disease.

The results of our feeding and inoculation experiments with these two species of sandflies, and other factors bearing on the transmission problem of Chinese Kala Azar, will be discussed in a future communication.

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The Development and Morphology of the Gonads of the Mouse.—Part I. *The Morphogenesis of the Indifferent Gonad and of the Ovary.*[*]

By F. W. Rogers Brambell, Fellow of the International Education Board.

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(From the Department of Anatomy (Embryology and Histology), University College, London.)

(Plates 28–31.)

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I.—*Introduction.*

The work described in this series of papers was carried out with a view to furnishing controls of the experiments on the mouse undertaken in collaboration with Dr. A. S. Parkes and others. This paper deals with the development of the indifferent gonad of the mouse, up to the stage at which it differentiates into an ovary or a testis. This takes place on the 12th day *post coitum*, when the testis can first be distinguished. The ovaries remain in the indifferent stage for some time longer, but can be identified by a process of elimination.

The present paper is concerned also with the development of the ovary from the time when sex can be distinguished until sexual maturity (8 weeks *post partum*). It is not proposed to consider here the structure of the maturing follicle, the process of atresia, or the formation of the corpus luteum. These problems will be reserved for a subsequent paper on the adult ovary of the mouse.

Details of the breeding, &c., of the colony of mice are recorded elsewhere (Parkes). Both copulation and parturition take place at night, the vaginal plug or litter, as the case may be, being detected the next morning. The average period between the findings of the plug and of the litter is 19 days and this may be taken as the normal gestation period for this colony.

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The ages of the embryos and young are calculated in the present series from the date of finding the plug or litter respectively, and several hours must be added on to arrive at the true age. Thus a 13-day embryo is from the uterus of a mouse killed on the morning of the 13th day after the finding of the plug, and 13½-day embryo from one killed on the evening of the 13th day. Actually, a few hours must be added on to each of these to arrive at the true age, if it is desired to do so. It was, however, found more convenient in practice to omit to do this. The young mouse is weaned at 3 weeks old. The young female attains sexual maturity at about 8 weeks old, and the young male at about the same age, but they continue to grow for some time afterwards.

The author desires to express his thanks to Prof. J. P. Hill, F.R.S., for his advice and criticism, and to Dr. A. S. Parkes for the animals used in this research.

2.—*Technique and Material.*

The embryos were fixed entire, with, in the larger ones, the abdomen opened to allow the fluid to penetrate, or the gonads were dissected out separately or attached to a portion of the lumbar body-wall. The ovaries were dissected out before fixing in the young animals. Complete serial sections were made of the region of the gonads of the smaller embryos, and of the ovaries themselves of the larger embryos and young animals.

The specimens were in the fixative in all cases before they had been dead more than a few minutes. The animals were killed by decapitation, without the use of an anæsthetic. Bouin's fluid was used, followed by Ehrlich's hæmatoxylin and eosin, or by Pasini's staining, or Champy's fluid followed by Heidenhain's iron-hæmatoxylin.

The material available consisted of 37 embryos from 18 pregnant mice, killed at daily or half-daily intervals from 9 to 18 days *p.c.* This material has been arranged in 17 Stages, as the variation in the state of development of the earlier embryos (9 to 12 days) necessitated a seriation not strictly in accordance with their ages. The variation in the stages of development between healthy embryos from the same uterus is always very small. The ovaries of 13 young animals, ranging from birth to maturity, were also available, and comprised Stages 18 to 28.

3.—*Description.*

The formation of the gonad.—The first indication of the formation of the germinal ridges is found in embryos 9 days *p.c.* (Stage 1), where a few of the so-called "primordial" germ-cells occur beneath the peritoneal epithelium along the ventral border of the Wolffian body. At this time the epithelium in this region is similar to that of the rest of the peritoneum and consists of a single layer of cells, but a few cells beneath its surface indicate the beginning of proliferation. This is soon followed by the thickening of the epithelium to form the germinal ridge (Stage 2, 9 days *p.c.*). The epithelium becomes two cells thick at first and continues proliferating to form a solid plate several cells thick along the ventral aspect of the Wolffian body. In section, this plate of cells is slightly concave on its dorsal side and convex on its ventral surface. It is first formed anteriorly and gradually develops in a posterior direction. It is formed from the covering epithelium by a proliferation of epithelial cells continuous over its surface, and not in the form of separate cords, blocks or chains of cells. The primordial germ-cells are included in this epithelial mass or "nucleus," which constitutes the germinal ridge. The peritoneal epithelium covering it is the germinal epithelium. Mitoses are numerous in Stages 2 to 9 in the cells of the germinal epithelium and epithelial nucleus.

The proliferation from the germinal epithelium continues, so that the resulting epithelial mass or nucleus changes in cross section from a flattened (Plate 30, fig. 5) to a broad oval and then to a circle (Stage 5, 10 days *p.c.*). This change begins anteriorly and gradually extends posteriorly. As soon as the epithelial nucleus has become circular in cross section at the anterior end the nipping off of the germinal ridge to form the Wolffian body and gonad begins. This nipping off is more apparent than actual, and results from the growth of the Wolffian body and gonad rather than from an actual deepening of the grooves which delimit them. The separation of the Wolffian body, with the gonad attached to it, is affected first (Plate 30, figs. 2 and 4). It begins anteriorly and extends gradually posteriorly (Stages, 6, 7 and 8; 11, 12 and 11½ days *p.c.* respectively) along the line where the dorso-lateral and ventro-medial grooves delimit the mesonephros. The Wolffian body is attached to the body-wall only by a relatively narrow mesentery (Plate 30, figs. 1 and 3) by the time sexual differentiation is definitely established (Stage 9, 12 days *p.c.*). The rapid growth of the gonad at this time results in the formation of two grooves along the dorsal margins of the epithelial nucleus, at first anteriorly. These extend posteriorly. This process results in the separation of the gonad from the Wolffian body, except for the hilar attachment. Nothing is included in the gonad but the epithelial nucleus, with its germ-cells and capillary loops, except a few mesenchymal elements in the angle between the dorsal aspect of the epithelial nucleus and the germinal epithelium on the median border at the extreme anterior end. The transformation of the germinal ridge into the definitive gonad is thus effected in a manner similar to that described in the human embryo by Felix (6).

The epithelial nucleus remains in complete continuity with the germinal epithelium until sexual differentiation takes place. The vascular supply is established from the beginning from the capillary loops which extend into the forming germinal ridge.

The differentiation of ovary and testis.—The differentiation of the indifferent gonad into a testis begins as soon as it is nipped off. The first indications of this process are seen in Stage 7 (12 days *p.c.*), and appear to set in between 11½ and 12 days *p.c.* They consist in the male (Plate 30, fig. 2) of a downgrowth of mesenchymal tissue from the base of the gonad between the germinal epithelium and the epithelial nucleus which begins anteriorly and gradually extends ventrally and posteriorly until it forms a continuous sheath beneath the epithelium. This is the primordium of the tunica albuginea of the testis. It becomes thickest along the ventral side of the gonad where the ends of the capillary loops extend into it. Other mesenchyme cells invade the epithelial nucleus, simultaneously with the formation of the albuginea, and break it up into thick twisted finger-shaped spermatic cords. The mesenchyme exhibits many mitoses. The downgrowth of mesenchyme results in the primordial germ-cells being confined to the spermatic cords. Numerous mitotic figures are present in the newly formed cords (Stage 9, 12 days *p.c.*) which grow rapidly. The formation of these spermatic cords from the proliferated epithelial cells is, therefore, secondary in the mouse. The germinal epithelium, isolated outside the newly formed albuginea, does not stop proliferating immediately, but continues for a short time (Plate 30, fig. 3), and becomes several cells thick (Stage 9, 12 days *p.c.*). The cells proliferated, however, never penetrate the tunica albuginea, but remain *in situ* and appear to become re-arranged as a single layer by growth and stretching.

The ovaries (Plate 30, fig. 4) do not develop characteristic structures until some time after the differentiation of the testes. In Stage 9 (12 days *p.c.*) they are still only negatively distinguishable by lacking any downgrowth of mesenchyme tissue or the formation of a tunica albuginea (Plate 30, fig. 1). The ovary (Stages 7, 8 and 9; 12, 11½ and 12 days *p.c.* respectively) remains in the indifferent condition with a solid epithelial nucleus everywhere continuous with the germinal epithelium. The epithelial nucleus undergoes no secondary rearrangement into cords by the downgrowth of connective tissue, as it does in the male. The germinal epithelium continues adding to it by proliferating cells. This proliferation in the female is an unbroken continuation of that which took place from the earliest formation of the germinal ridge.

Sex cannot be determined by dissection under a binocular dissecting microscope at 12 days *p.c.* (Stage 9). At 12¼ days *p.c.* the testes appear rather thicker than the ovaries and this difference is more marked at 12½ days *p.c.* At all subsequent stages sex can easily be determined by dissection.

The primordial germ-cells.—The so-called primordial or primitive germ-cells of other authors occur in the mouse. A few are present in Stage 1 (9 days *p.c.*) in the region of the germinal ridge, but are not found elsewhere. These cells (Plate 28, figs. 1, 6 to 8) are characterised by their large size and clear appearance, owing to staining less densely than the surrounding cells. The nuclei are large and oval, not irregular in shape, and measure 8·5 μ in average diameter. They contain two or three large acidophil nucleoli and a small amount of chromatin, chiefly scattered around the nuclear membrane and nucleoli in small lumps and granules of irregular size and shape. Linen threads, with fine chromatin granules strung on them, radiate from the largest nucleolus, which is situated near the centre of the nucleus, to the larger lumps of chromatin around the periphery. It is impossible to determine whether these threads are attached to the nucleolus itself or to the fragments of chromatin clustered close around it. In general, the nucleus is large and clear, with little chromophil material compared with the other cells of the embryo.

The cells of the germinal epithelium (Plate 28, fig. 2) at the stages until sexual differentiation is definitely established, resemble in many points the primordial germ-cells (Plate 28, fig. 7). Like them, the nuclei contain two, three or more large acidophil nucleoli. The chromatin is rather more evenly distributed, but strings of small granules can sometimes be seen radiating from the nucleoli to the larger peripheral lumps of chromatin. The nuclei probably contain about the same amount of chromophil material as those of the primordial germ-cells, but they appear much denser, owing to their smaller size and the consequent greater concentration of the chromatin. The cytoplasm is also rather more deeply staining than in the primordial germ-cells. The cell-body and the nucleus are smaller, and the latter is less regular in shape. Cells, similar to those in the germinal epithelium, are plentiful in the epithelial nucleus (Plate 28, fig. 3).

Every gradation of cell intermediate in character (Plate 28, figs. 3, 5) between these and the primordial germ-cells is also present in the epithelial nucleus (Plate 29, fig. 2) of this and other stages prior to sexual differentiation. It is impossible in many cases to decide whether a given one of these cells should be considered a primordial germ-cell or an epithelial cell. Other cells in the epithelial nucleus of the gonad are more irregular in shape and size with irregular nuclei (Plate 28, fig. 4). These nuclei contain several scattered acidophil nucleoli and are rich in chromatin, which is scattered throughout, making them appear very dense and in marked contrast to those of the germ-cells. These cells are obviously of epithelial origin also, and stages intermediate between them and the cells of the germinal epithelium are present.

Some of the primordial germ-cells, especially about Stage 7 (12 days *p.c.*), are unusually large (Plate 28, fig. 6). Mitoses occur in the primordial germ-cells from Stage 2 (9 days *p.c.*); they are frequent especially from Stage 5 (10 days *p.c.*) onwards.

It is noteworthy that the primordial germ-cells are confined to the germinal ridge and are absent from the mesentery of the gut and other parts of the embryo in Stage 1 (9 days *p.c.*). In Stage 9 (12 days *p.c.*) and all subsequent stages they are also confined to the gonad. In Stages 2 to 8 inclusive they occurred outside the germinal ridge (Plate 29, fig. 1). The distribution of these extra-regional germ-cells is summarised in Table I.

The development of the ovary.—The germinal epithelium:—The early proliferation of cells from the germinal epithelium continues for some time without intermission in the ovary after sexual differentiation. This is not so in the male, in which the proliferation ceases soon after sexual differentiation and the establishment of the tunica albuginea. The proliferation is virtually at an end in the 12-day *p.c.* male, but it is still actively progressing in the 12½-day female. In the female from this time onwards the proliferation becomes less active, but continues until puberty. The cells are not proliferated from the epithelium in the form of separate strands or cords, but as single cells more or less continuously over the surface until the septa ovarii have extended to the periphery. From this time the cells are proliferated between the septa, in the form of thick cords. This corded arrangement of the proliferation is secondary and is imposed by the development of the septa. The cells, as they become separated from the epithelium, push their way through the thin tunica albuginea where it is present and merge with the other elements of the ovarian cortex. The proliferation appears rather more active at first on the side of the ovary away from the mesovarium. After birth the proliferation becomes less evident, and after puberty is attained at about 7 weeks *p.p.*, it

Table I.

Stage.	Distribution of Extra-regional germ-cells.	Stage of development of gonads.
1 (9 days *p.c.*)	None	First rudiment of germinal ridge forming.
2 (9 days *p.c.*)	Some in immediate vicinity of the base of the germinal ridge.	Germinal ridge formed.
3 (11 days *p.c.*)	Near base of germinal ridge and one in the upper part of the mesentery.	Germinal ridge formed.
4 (12 days *p.c.*)	Plentiful near the base of the germinal ridge.	Lateral grooves beginning to deepen anteriorly.
5 (10 days *p.c.*)	Plentiful near the base of the germinal ridge, many in the mesentery and some far down it.	Nipping off begun, but is not complete even at the anterior end.
6 (11 days *p.c.*)	Near the base of the germinal ridge only.	Nipping off more advanced.
7 (12 days *p.c.*)	Near the base of the germinal ridge at the posterior end only.	Nipping off complete at the anterior end and proceeding at the posterior end.
8 (11½ days *p.c.*)	An occasional one near the base of the germinal ridge.	Nipping off almost complete, even at the posterior end.
9 (12 days *p.c.*)	None	Nipping off of the gonads complete.

has apparently ceased altogether. The small amount of *post-partum* proliferation that seems to occur is limited to the regions between the larger follicles and is more active in the neighbourhood of the hilum.

The epithelial proliferation in the mouse is thus one single process continuing without intermission from the time when the germinal ridge is first formed at 9 days *p.c.* until about the seventh week *p.p.* when puberty is attained. The present material affords no evidence justifying the division of this process in the mouse into medullary, cortical and definitive proliferations, such as de Winiwarter and Sainmont have demonstrated in the ovary of the cat (16).

The epithelial nucleus.—The epithelial nucleus of the ovary is at first composed entirely of epithelial cells and germ-cells. Numerous mitoses are observable in both, and the growth of the whole is contributed to by the continued proliferation of more epithelial cells from the germinal epithelium. Subsequently to the downgrowth of mesenchymal elements into the epithelial nucleus, which begins soon after sex is established, the cells of epithelial origin remain distinguishable as such. They continue to increase, but more slowly, by division within the ovary and by further proliferation from the germinal epithelium. These epithelial elements are observable in the cortex of the young ovary and are an important constituent of the inter-follicular tissue. The intermediate stages described above in the indifferent gonad, suggestive of the formation of germ-cells from the epithelial cells, are present in the ovary after sexual differentiation and are observable until all the germ-cells have entered on the prophase changes about the 13th or 14th day *post coitum*.

The germ-cells.—The "primordial" germ-cells found in the gonad from the time of its formation exhibit many mitoses until the 12th day *p.c.*, after which they multiply less rapidly, and by the 13th or 14th day *p.c.* have ceased dividing. At this time all the germ-cells in the gonad enter upon the prophase changes and subsequently none can be found with resting nuclei. There is little degeneration of germ-cells observable in the gonad before sexual differentiation or in the ovary until about the 16th day *p.c.* All the germ-cells are similar and there are no cytological grounds for distinguishing the so-called "primordial" from the later formed germ-cells. It would appear, therefore, leaving aside the question of their origin, that the so-called "primordial" germ-cells in the mouse persist and enter on the reduction stages.

Some early prophase stages can be distinguished as early as Stage 9 (12 days *p.c.*), but it is not till a day or two later (Stages 12 and 13, 13½ and 14 days *p.c.*) that all the germ-cells exhibit them (Plate 31, fig. 4). Deutobroque stages are most plentiful at this time. Synaptic stages become most plentiful about 15 or 16 days *p.c.* (Stages 14 and 15). In the new-born young (Plate 31, fig. 3) there are no stages earlier than pachytene, which are scarce. Many of the nuclei are diplotene, but the majority are already dictyate. In the 4-day old young (Stage 19), all the oocytes have reached the dictyate condition. From this time on, no reduction stages earlier than the dictyate are observable in any of the oocytes of the mouse.

Germ-cells are found actually in the germinal epithelium of the ovary at all stages after sex is differentiated and in the adult. Prior to the appearance of the prophase changes the nuclei of these are in the resting stage or in mitosis. From the 13th or 14th day *p.c.* onwards they always exhibit prophase stages like the germ-cells in the deeper parts of the ovary. In the young mouse, four or more days old, and throughout later life these oocytes in the epithelium are always in the dictyate condition and never exhibit the earlier prophase stages.

The tunica albuginea and septa ovarii.—The ovary is composed, as already described, of an epithelial nucleus consisting of germ-cells and epithelial elements alone, which is everywhere continuous with the germinal epithelium at 12 days *p.c.* (Stage 9). No primitive tunica albuginea is developed in the mouse ovary, such as has been described in other mammals about this stage of development. The only mesenchymal tissue present in the mouse ovary at this time is a slight downgrowth between the epithelial nucleus and the germinal epithelium along the median dorsal border at the extreme anterior end. This band of mesenchyme is never extensive and appears to have been included in the gonad by the processes resulting in its separation, with the Wolffian body, from the body wall. It is impossible to say whether or not this structure represents the rudiments of the primitive tunica albuginea of other forms.

The downgrowth of mesenchymal elements from the hilum, which gives rise to the connective tissue of the ovary and which results in the formation of the definitive tunica albuginea and septa ovarii, begins shortly after the differentiation of sex. The first indications of this downgrowth can be detected at 12½ days *p.c.* (Stage 10), especially at the anterior end. They consist of the penetration of a few elements from the mesenchyme of the hilum between the germinal epithelium and the epithelial nucleus along the medial and lateral dorsal borders of the ovary, and of others into the epithelial nucleus itself along the mid-dorsal line. The peripheral downgrowths constitute the primordium of the tunica albuginea, and the trabeculæ extending into the epithelial nucleus represent the primordia of the septa ovarii.

The tunica albuginea gradually extends ventralwards and at the same time develops posteriorly. About the 16th day *p.c.* (Stage 15) it becomes continuous over the ovarian surface under the germinal epithelium. It is, however, very thin at this time and consists of a single layer of mesenchyme cells. It gradually becomes more definite and thicker and its cells develop into connective tissue.

At the same time, the septa ovarii extend radially from the hilum towards the periphery of the ovary (Plate 31, fig. 4). Some reach the periphery and fuse with the tunica albuginea about Stage 12 (13½ days *p.c.*), and, by the time the tunica albuginea is continuous, they are completely established. They consist at first of thin trabeculæ of mesenchyme cells growing down from the hilum. Later these transform into connective tissue. The septa ovarii break up the epithelial nucleus, previously a solid mass, into thick finger-shaped cords radiating from the hilar region to the periphery (Plate 31, figs. 2 and 3). At the time the rete tubules become united with the hilar insertions of these epithelial cords, when they are being formed by the development of the septa ovarii, and the urogenital union is effected. The bases of the septa ovarii, in consequence, form sheaths around the connections of the rete and epithelial cords.

These sheaths, which become augmented by the further downgrowth of mesenchymatous elements from the hilum, constitute the mediastinum ovarii.

The medulla.—The formation of the medulla results from two simultaneous processes: (*a*) the degeneration of the germ-cells in the deepest parts of the epithelial cords, and (*b*) the downgrowth of connective tissue from the hilum. The first indications of medulla formation are observed at 16 days *p.c.* (Stage 15), when a number of the central germ-cells exhibit signs of degeneration and the connective tissue forming the mediastinum and the bases of the septa ovarii is becoming thickened. At the next stage (17 days *p.c.*) the medulla forms a well marked knob of tissue projecting into the ovary from the hilum (Plate 31, fig. 2). The downgrowth of connective tissue from the hilum and the degeneration of the oocytes in the deeper layers of the epithelial nucleus are well marked. The development of the medulla continues and in the new-born young is more or less complete (Plate 31, fig. 3).

The medulla is therefore formed around the rete connections and the blood-vessels of the ovary. The rete tubules remain in it as more or less constant remnants of the normal ovary throughout life.

The extensive degeneration of large follicles, occurring in the ovary at weaning time, results in an extension and consequent loosening of the medullary zone. The loose medulla of the adult ovary contains many lacunæ and enlarged blood vessels.

The follicles.—The larger oocytes, situated in the inner ends of the epithelial cords, first develop a follicular epithelium. Some of these have three or four flattened epithelial cells grouped around them at 18 days *p.c.* (Stage 17). These follicle cells are derived from the undifferentiated epithelial elements of the cords and they constitute the primordium of the membrana granulosa. The number of these primitive follicles increases rapidly until at 6 days *p.p.* (Stage 20) all the oocytes have a follicular epithelium. The formation of the follicles is speedily followed by the growth of the deeper ones. The oocytes of these having dictyate nuclei enter upon the growth stage and the follicular epithelium grows into a layer of cubical cells, at first single, then two or three cells thick. Numerous mitoses are observable in the cells of the growing follicular epithelium. Many such follicles are already apparent at 6 days *p.p.* (Stage 20). Meanwhile, each of these first formed follicles has acquired a primitive theca. The primitive theca is forming around some of the follicles at 4 days *p.p.* (Stage 19). It is composed of a thin coat of elements, like connective tissue cells, arranged in a circular manner around the follicular epithelium. It is difficult to be sure whether these elements are derived from the epithelial cells of the cords or from the connective tissue cells of the septa ovarii and medulla. Each follicle, by the time the membrana granulosa consists of a single layered cubical epithelium, is provided with such a primitive thecal coat.

A very large number of follicles enter on the growth stage immediately after their formation. Many in process of rapid growth can be seen at 6 days *p.p.* (Stage 20). The oocyte grows to mature size and the membrana granulosa becomes many cells thick (Stages 21 and 22; 8 and 14 days *p.p.*). Traces of an antrum are seen in some of the largest follicles at 14 days *p.p.* (Stage 22). These growing follicles reach their maximum development at weaning time (3 weeks *p.p.*, Stage 23). At this stage these follicles compose the major part of the ovary (Plate 31, fig. 1), which is as large or even larger than the adult organ, having increased enormously in size, owing to the growing follicles, during the previous fortnight. As many as fifty such precocious follicles can be counted in a single section at this stage. The largest follicles measure about 0·3 mm. in diameter and have a distinct antrum. The oocytes in the majority of these follicles exhibit obvious signs of degeneration: they are vacuolated and slightly shrunken and often exhibit spindles, polar bodies and even cleavage stages; phenomena well recognised in the oocytes of degenerating follicles. They sometimes contain crystalloidal bodies, obviously also degeneration products. The membrana granulosa of the follicles also exhibits signs of degeneration in the large number of pycnotic cells and the invasion of leucocytes.

The appearance at the next stage (4 weeks *p.p.*, Stage 24) is very different. The majority of the precocious follicles have degenerated and disappeared, in consequence of which the ovary has shrunken considerably in size and its surface has become somewhat folded. The medullary zone has extended, and become loosened in texture, doubtless owing to the sudden slackening of the internal pressure exerted by the follicles. The degenerate remains of some of the oocytes and follicles are present and an occasional full-grown oocyte, with only a single layered follicular epithelium stretched thin over its surface. How such a follicle could have supplied sufficient nourishment for the oocyte to attain mature dimensions is a problem, yet this abnormality is by no means uncommon in the ovary of the mouse one month or more old. In this, as in all subsequent stages, a number of growing follicles of various sizes occurs, although they are never as numerous as in the previous stage (23; 21 days *p.p.*). Some of these are apparently normal, others obviously degenerating. The size of the largest follicles present increases until maturity is attained, usually about the 8th week *post partum* (Stage 28), when ovulation occurs and the œstrous cycle starts.

I have not observed follicular atresia resulting in the formation of corpora lutea atretica in the mouse ovary prior to the attainment of sexual maturity. This may be associated with the fact that there is no "interstitial" tissue composed of large vacuolated cells, such as occurs in the rabbit and the cat, and which has been said to originate from the cells of the corpora lutea atretica by some authors, in the normal ovary of the mouse.

The degeneration and absorption of the oocyte results in a cavity being formed in the follicle around the shrunken zona pellucida, the most persistent part of the oocyte. In many cases the membrana granulosa breaks down into this cavity and degenerates completely and the theca shrinks in size. This process results in the former follicle being only represented by a small cavity, containing the crumpled zona pellucida of the oocyte, surrounded by a fibrous wall, the old theca. In some follicles, however, after the breakdown of the oocyte, fibroblasts from the theca appear to penetrate the membrana granulosa and form a wall within it around the cavity containing the remnants of the oocyte. In this case the cells of the membrana granulosa may persist and add to the epithelial elements composing the interfollicular tissue of the ovarian cortex. Such cells, however, do not form large vacuolated interstitial cells like those found in the ovary of the cat and the rabbit.

4.—*Discussion.*

The "primordial" germ cells.—In 1870 Waldeyer (14) expressed the view that the germ cells arose from the germinal epithelium. Five years later Goette (7) suggested the extra-regional origin of the germ-cells in the toad and their possible migration. Many authors, including Rubaschkin (13), have supported this view, but Dustin (5) suggested a dual origin for the germ-cells, and almost simultaneously de Winiwarter and Sainmont (16) maintained that the primordial germ-cells all degenerate in the cat, and that the definitive germ-cells arise later from the germinal epithelium. De Winiwarter (17) later insisted on a similar fate for the primordial germ-cells in the human being. Felix (6), expressed the view that in man the primordial germ-cells disappeared and that the secondary germ-cells arose from the germinal epithelium. In the mouse, Jenkinson (8) figured primordial germ-cells and described them as forming in the yolk-sac and migrating to the genital ridge. He was cautious in expressing an opinion as to their fate, and admitted the possibility of an epithelial origin for some of the germ-cells.

There can be no doubt that the so-called "primordial" germ-cells of other authors occur in the mouse. They are present in the primordium of the germinal ridge before the outset of the epithelial proliferation (Stage 1; 9 days *p.c.*), and are found in the mesentery of the gut and in the mesenchyme near the germinal ridge, as well as in it, until the nipping off of the gonad is completed. Their cytological characteristics render them easily distinguishable from the other cells of the body and they are indistinguishable from the germ-cells that, in later stages, undergo the prophase changes of the reduction division. Further, there is no evidence, in the material studied, that these undergo degeneration; on the contrary, they would appear to persist and to undergo the maturation phases.

The occurrence of primordial germ-cells outside the germinal ridge, from the time when it is formed until the gonad is nipped off, is certain also. In the mouse they are found first in the forming germinal ridge (Stage 1; 9 days *p.c.*). Subsequently, they are found in the mesenchyme at the base of the germinal ridge (Plate 29, fig. 1), between it and the base of the mesentery of the gut and even some distance from the latter. They appear to be absent, however, from all other parts of the embryo, and in the regions referred to their frequency greatly increased as the distance from the germinal ridge decreased. They were not found outside the gonad shortly after the latter was completely nipped off (Stage 9; 12 days *p.c.*). It would appear probable that the primordial germ-cells are (*a*) either formed at a distance from the germinal ridge and migrate up the mesentery and through the mesenchyme of the dorsal body-wall to it, as described by many authors, or are (*b*) formed in the germinal ridge and wander out of it. The distribution described in this paper (Table I) is in favour of either hypothesis. The apparent formation of germ-cells from epithelial cells in the germinal ridge, and the failure to find a yolk-sac origin for the extra-regional germ-cells, however, support the latter suggestion, although the material described is insufficient to arrive at a conclusion.

It remains to consider the evidence for the formation of germ-cells within the germinal ridge. The cytological description indicates that the cells of the germinal epithelium which are proliferated into the germinal ridge may differentiate in two different directions: (*a*) into germ-cells; (*b*) into cells with irregular-shaped dense nuclei which will presumably form the folliclar epithelium. Every intermediate stage between the undoubted germ-cells and the undifferentiated germinal epithelial cells can be seen in a single section and typical examples are figured (Plate 28 and Plate 29, fig. 2). The cytological evidence for the formation of the "primordial" germ-cells in the germinal ridge is, therefore, satisfactory. Furthermore, even if it were established that the primordial germ-cells migrate into the germinal ridge from elsewhere it would still be impossible to deny that others, in every way indistinguishable from them, were differentiated from the epithelial cells. It would also be difficult to imagine a sufficient number of germ-cells migrating to account for the rapid increase in the number found in the gonad between 10 and 12 days *p.c.*, although many of those in the gonads are in mitosis. It is worthy of note that no marked degeneration of germ-cells in the gonads is observable up to the time when sex is definitely established.

The later formation of germ-cells.—De Winiwarter and Sainmont (16) find that all the germ-cells of the medullary and cortical cords degenerate in the cat, and that the definitive oocytes are formed from about 4 months *p.p.* They describe at great length the nuclear changes observable in the ovules of the second proliferation or cortical cords. They state that they pass successively through the protobroque, poussieroïde, deutobroque, leptotene, synaptene, pachytene, diplotene and dictyæ stages. They state at the end that none of these cells ever produce the definitive ova, all of which are derived from the third proliferation.

Kingery finds that in the mouse (9) all the germ-cells formed before birth degenerate. The second formation of germ-cells, the definitive oocytes, from the germinal epithelium is most rapid from 3 to 25 days *post partum*, but goes on slowly almost until sexual maturity at 40–45 days after birth. The definitive germ-cells pass in from the epithelium, through the tunica, singly, not in cords. Kingery states that all the oocytes which will be differentiated are present in the ovaries when sexual maturity is attained. He says that "*in the development of the definitive germ-cells, which are formed after birth, there is not the slightest indication of synizesis.*"

Germ-cell counts made at different ages would be, obviously, the most direct method of solving these problems. The enormous labour involved in making a sufficient number of counts of each stage to produce significant figures, in view of the large individual variation and the inaccuracy of the counts, renders this almost impossible,

The material described in this paper shows that the nuclei of all the oocytes have reached the dictyate stage shortly after birth (4 days *p.p.*). Subsequently the earlier reduction figures are never found in any of the germ-cells. Therefore if any of the germ-cells are formed after birth it is clear that they do not pass through these prophase changes. The occurrence of small oocytes in the germinal epithelium at all stages does not necessarily prove, although it suggests, their formation there. Such oocytes may have remained there during the development of the ovary, or they may have been pushed into the epithelium from the cortex by the internal pressure exerted by the growth of the deeper-lying follicles.

The present material also appears to show that many of the earlier formed germ-cells do persist until adult life. Although a certain number of oocytes degenerate before birth and a large number enter on a precocious growth stage after birth and degenerate about 3 weeks *post partum*, many oocytes in various stages remain and appear to persist. The degeneration of oocytes about weaning time corresponds to the *post-partum* degenerations noticed by de Winiwarter and Sainmont (16) and by Kingery (9), and which they believed involved all the earlier-formed germ-cells. It appears from some experimental results recorded elsewhere in collaboration with Dr. Parkes and Miss Fielding (4) that germ-cells present in the ovary before birth can persist until adult life. In these experiments mice were X-rayed *in utero* and at birth. The majority of the oocytes in these animals degenerated speedily, but some remained and developed a follicular epithelium. These follicles grew and persisted until about the time when the animals became mature, when they ultimately became atretic. It is noteworthy that the ovaries of these mice sterilized at or before birth, as well as of others sterilized by X-rays at weaning time (3), never regenerated any germ-cells although they remained functional in regulating the œstrous cycle. On the other hand, regeneration of ovarian tissue, *containing germ-cells*, after complete double ovariotomy has been shown to occur (12) in about 10 per cent. of cases in the mouse. In view of these apparently conflicting results, it is impossible to arrive at a definite conclusion as to whether there is a *post partum* formation of germ-cells in the ovary of the mouse.

The differentiation of sex.—The gonads of the mouse differentiate into ovaries or testes very early. It is possible to distinguish histologically the testes at 11½ or 12 days *p.c.* The ovaries can then be distinguished by a process of elimination, but do not exhibit characteristic ovarian structures until some hours later. This differentiation of the testes before the ovaries has been observed in the cat by de Winiwarter and Sainmont (16) and in the cow by Van Beek (2). Sex can be distinguished microscopically at 12½ days *p.c.* Kirkham (11), however, failed to distinguish sex earlier than 13 days *p.c.* in the mouse embryo.

The differentiating testis develops a tunica albuginea, which separates the spermatic cords from the epithelium, as described in the cat by de Winiwarter and Sainmont (16). The ovary, however, develops no corresponding primitive tunica albuginea and the epithelial nucleus remains continuous with the germinal epithelium. De Winiwarter and Sainmont (16) in the cat, and Van Beek (2) in the cow, describe the formation of a transient tunica albuginea, and Felix (6) in the human being finds a separation of the epithelial nucleus from the superficial epithelium at the time of the differentiation of sex.

The epithelial proliferations.—The proliferation from the epithelium which forms the epithelial nucleus of the indifferent gonad of the mouse continues without interruption, although more slowly, in the ovary throughout embryonic life and after birth until weaning time or even later. The fact that, in the male, the germinal epithelium continues proliferating for a brief period after the establishment of a continuous tunica albuginea, is further evidence that the proliferation is one continuous process. The proliferation takes place over the whole surface of the ovary and is not, at first, in the form of separate cords. Later the formation of the septa ovarii breaks it up into thick cords.

De Winiwarter and Sainmont in the cat (16), and the former author in the rabbit (15) and man (17), find three distinct epithelial proliferations in the form of cords: the medullary, cortical and third or definitive proliferations. Kingsbury in the cat (10) and Kingery in the mouse (9) fail to find any distinction between the medullary and cortical cords, but both distinguish a *post-partum* formation of germ-cells. Allen (1) describes the formation from the epithelium of medullary and Pflüger cords in the ovaries of embryo rabbits and pigs. Van Beek (2) in the cow finds that the medullary and cortical cords do not arise from a separate epithelial proliferation, but he also distinguishes a separate *post-partum* formation of germ-cells. The present results are, therefore, most nearly in agreement with those of Kingery, but do not show any distinction between the embryonic and the *post-partum* proliferation. The lack of a primitive tunica albuginea in the ovary, like that described in other mammals, is probably correlated with the absence of a break between medullary and cortical proliferations. These peculiarities of the ovary of the mouse may be due to the small size and extremely rapid development of the organ.

5.—*Summary.*

1. The first traces of the germinal ridge are found in the 9-day *p.c.* mouse embryo.

2. The growth of the germinal ridge is effected by proliferation from the epithelium. This results in the formation of a solid epithelial nucleus, which is not in the form of separate cords.

3. The testes differentiate from $11\frac{1}{2}$ to 12 days *p.c.* The differentiation is effected by the downgrowth of connective tissue from the hilum forming a continuous tunica albuginea and breaking the epithelial nucleus up into twisted spermatic cords. The formation of the spermatic cords is therefore secondary.

4. The ovaries remain in the indifferent condition after the testes have differentiated. There is no downgrowth of connective tissue, no primitive tunica albuginea and the epithelial nucleus remains a solid mass not broken up into cords. The epithelium continues proliferating without intermission.

5. " Primordial " germ-cells are found in the mouse. Evidence is brought forward, suggesting that they originate from the cells of the germinal epithelium and that some, wandering out of the gonad, constitute the extra-regional primordial germ-cells.

6. In the ovary the epithelial proliferation continues, without intermission, although more slowly, until the 4th or 5th week *post partum*. This proliferation cannot be divided into medullary, cortical and definitive proliferations, as described by de Winiwarter and Sainmont in the cat (16).

7. Mesenchymal elements growing down from the hilum form the tunica albuginea and septa ovarii. These are completely established by 16 days *p.c.* The development of the septa ovarii imposes a secondary corded arrangement on the epithelial nucleus of the ovary.

8. The medulla is formed by a downgrowth of connective tissue from the hilum at the time when the deeper situated germ-cells are degenerating.

9. Some oocytes can be seen degenerating at all stages after the differentiation of the ovary. Shortly after birth a large number of follicles enter upon a precocious growth stage. These reach their maximum development and then degenerate about weaning time (3 weeks *p.p.*).

10. No conclusion was arrived at as to whether there was a *post-partum* formation of oocytes in the young mouse. No germ-cells exhibiting the synaptic stages were observed in the stages more than four days old.

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DESCRIPTION OF PLATES.

GUIDE LETTERS :—*B.V.*, Blood vessel. *G.E.*, Germinal epithelium. *G.E.* 1–3, Stages in transformation of germinal epithelial cells into germ-cells. *G.M.*, Germ-cells in mitosis. *M.*, Medulla. *M.T.*, Mesonephric tubule. *M.D.*, Müllerian duct. *P.G.*, Primordial germ-cell. *R.T.*, Rete tubule. *S.O.*, Septa ovarii. *T.A.*, Tunica albuginea. *U.E.*, Undifferentiated epithelial cell in epithelial nucleus. *W.D.*, Wolffian duct. The arrow points to the mid-line of the embryo in all cases.

PLATE 28.

FIG. 1.—Primordial germ-cell from 11½-day embryo. × 2340.

FIG. 2.—Three cells of the germinal epithelium of 11½-day embryo. × 2340.

FIG. 3.—Cell in epithelial nucleus of 11½-day embryo illustrating first stage in transformation of an epithelial cell into a germ-cell. × 2340.

FIG. 4.—Two undifferentiated epithelial cells in the epithelial nucleus of 11½-day embryo. × 2340.

FIG. 5.—Two cells in the epithelial nucleus of 11½-day embryo, illustrating the second stage in the transformation of an epithelial cell into a germ-cell. × 2340.

FIG. 6.—Very large primordial germ-cell from a 12-day embryo. × 2340.

FIG. 7.—Primordial germ-cell from 9-day embryo. × 2340.

FIG. 8.—Primordial germ-cell and mesenchyme cell from a 9-day embryo. × 2340.

PLATE 29.

FIG. 1.—Six primordial germ-cells in the body-wall above the germinal ridge of a 10-day embryo. × 1000.

FIG. 2.—Germinal ridge of a 10-day embryo showing cells proliferated from the germinal epithelium in the undifferentiated condition and in various stages of differentiation into germ-cells. × 1000.

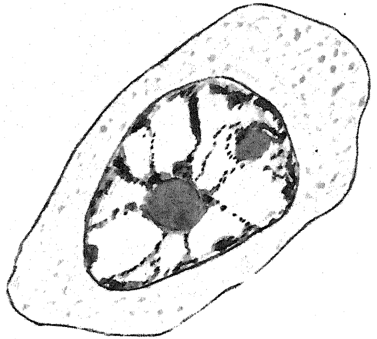
PLATE 30.

FIG. 1.—Ovary of 12-day embryo (Stage 9), showing epithelial nucleus everywhere continuous with the germinal epithelium. × 240.

FIG. 2.—Testis of 12-day embryo (Stage 7), showing developing tunica albuginea and germinal epithelium thickened outside it. × 280.

FIG. 3.—Testis of 12-day embryo (Stage 9), showing spermatic tubules cut off from the germinal epithelium by a well developed tunica albuginea. × 170.

FIG. 4.—Ovary of 12-day embryo (Stage 7), showing epithelial nucleus still continuous with the germinal epithelium. × 280.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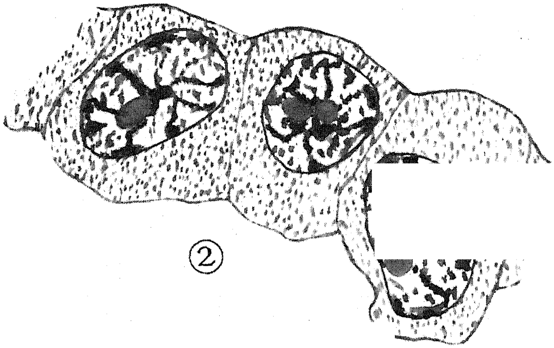
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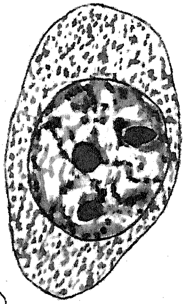
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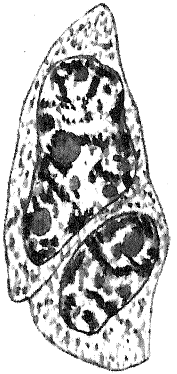
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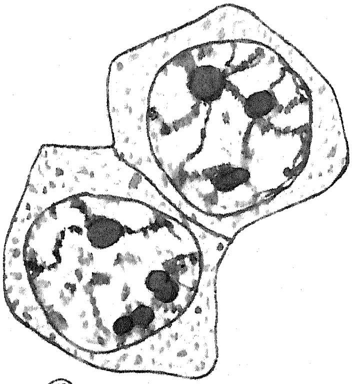
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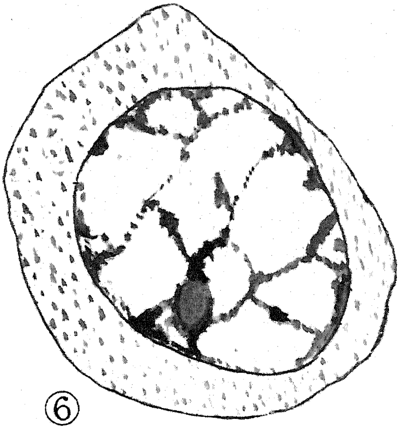
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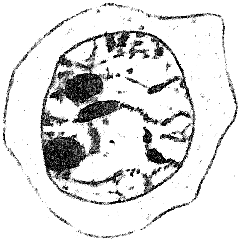
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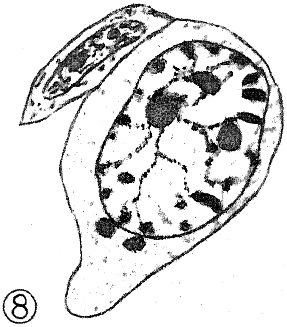
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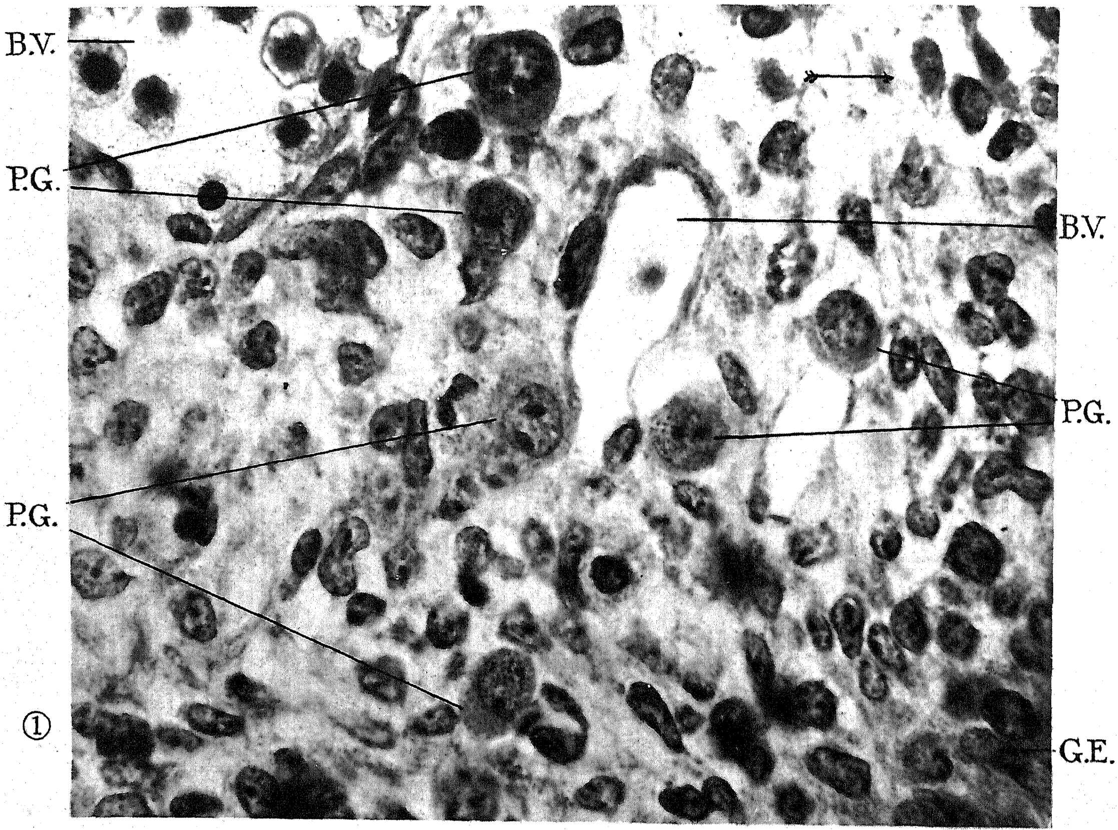

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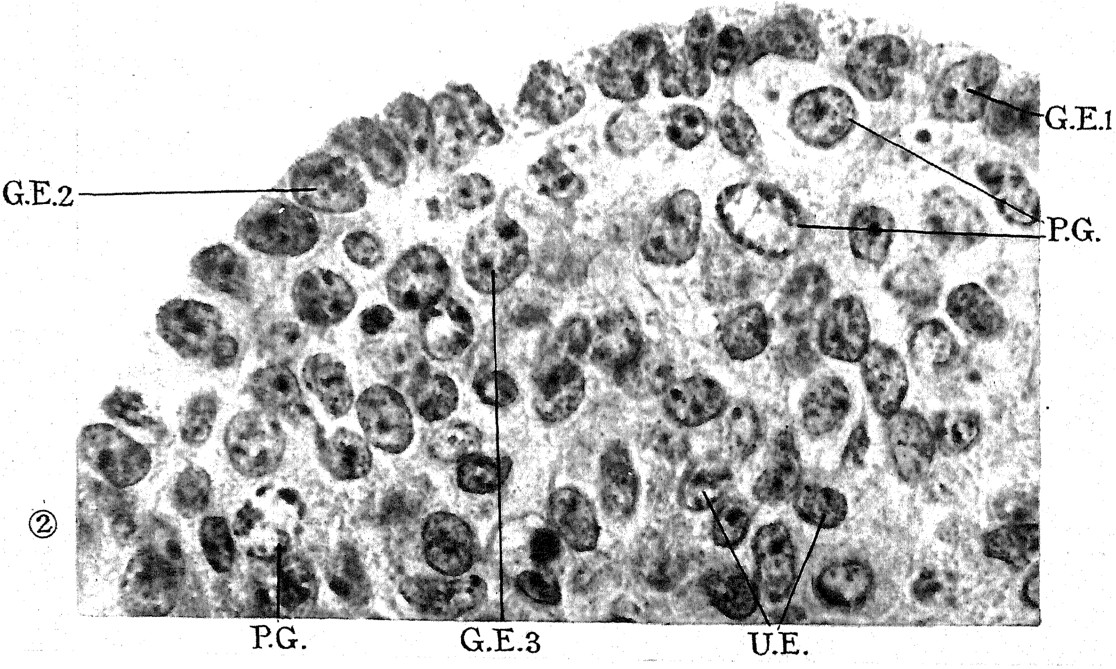

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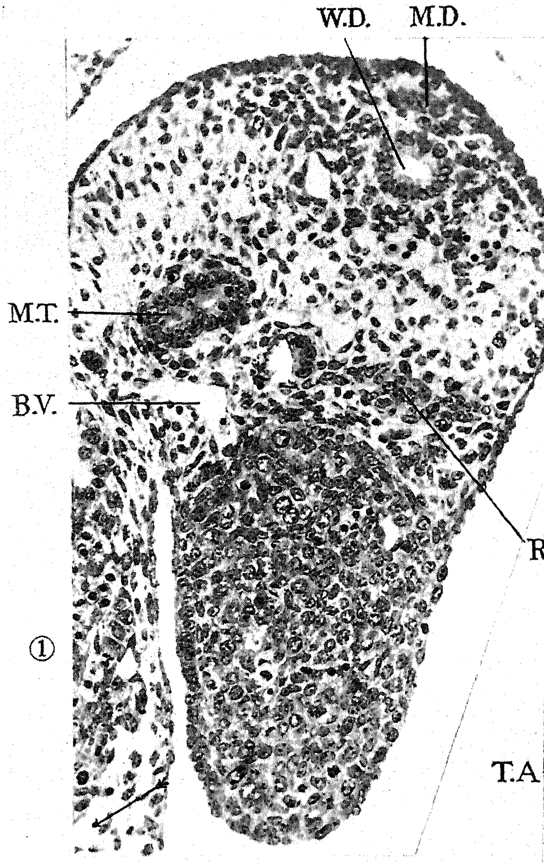

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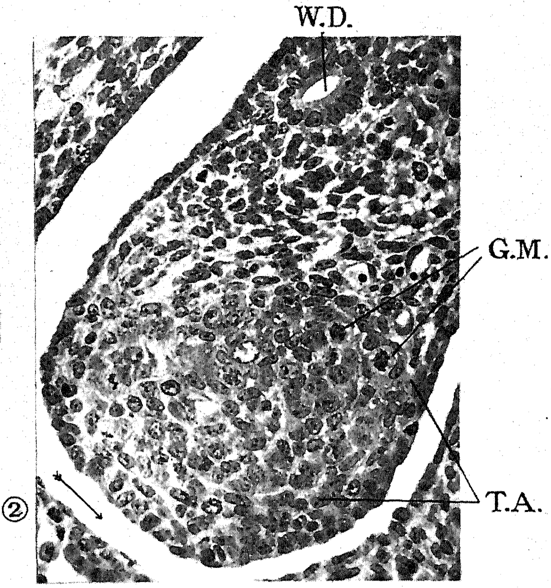

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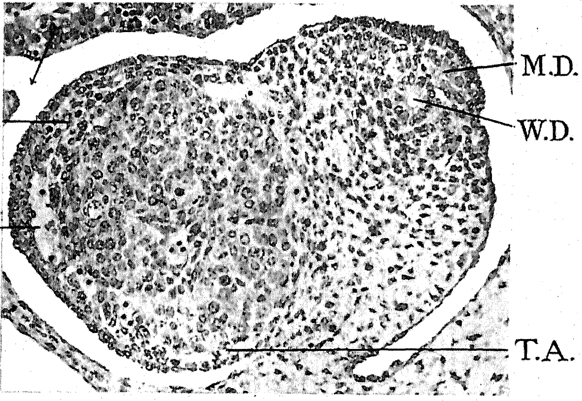

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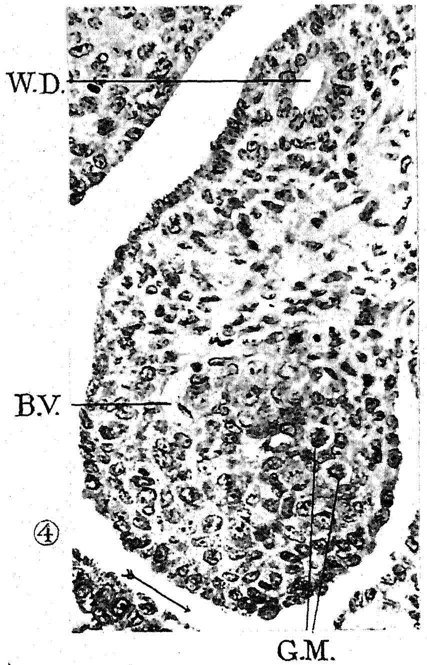

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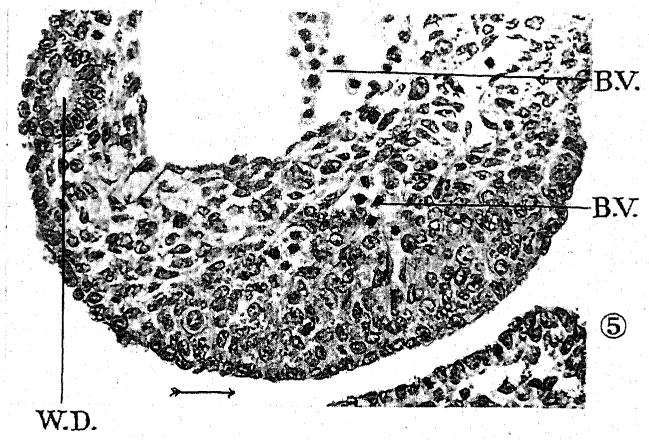

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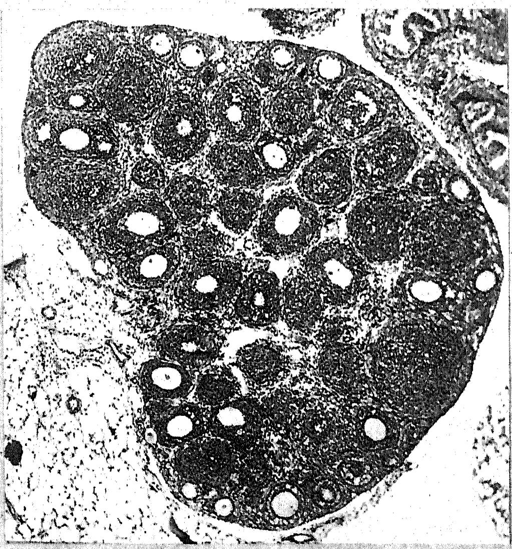

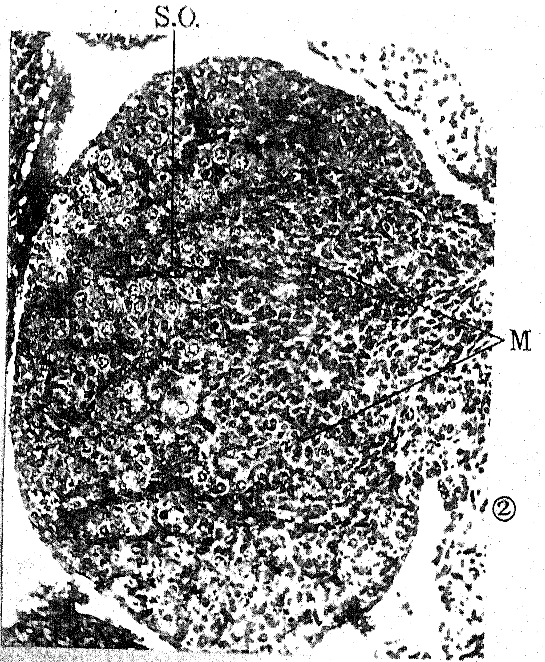

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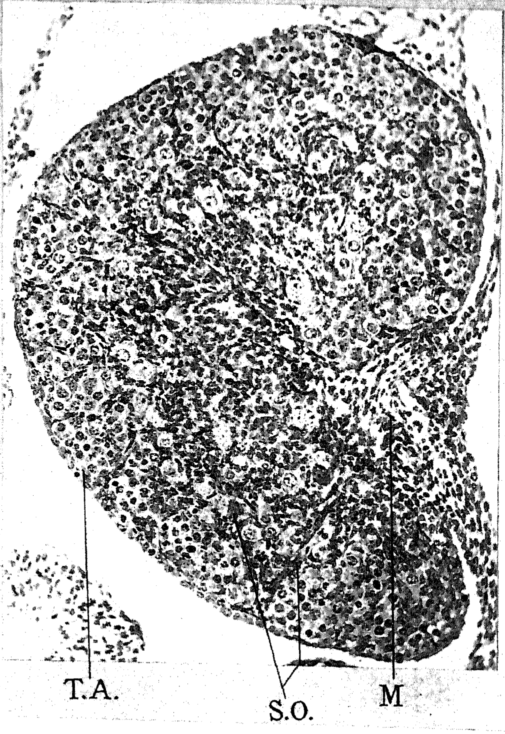

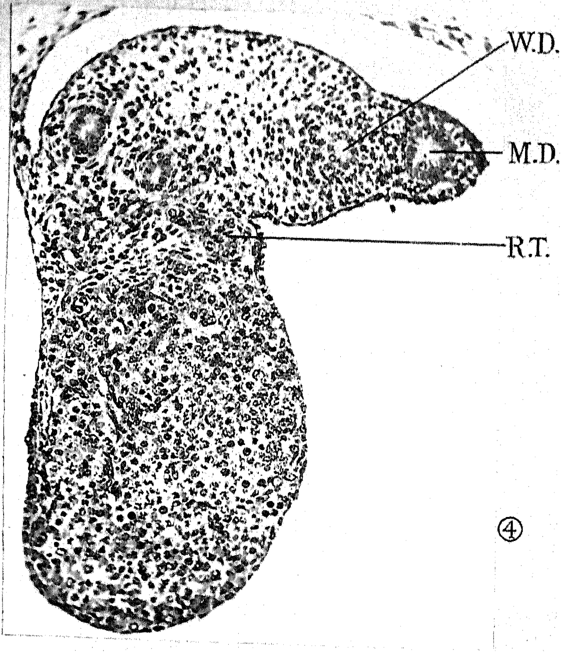

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Fig. 5.—Germinal ridge of 12-day embryo (Stage 4), showing formation of epithelial nucleus. × 265.

Plate 31.

Fig. 1.—Ovary of young mouse three weeks old, showing large number of follicles which have developed precociously and are about to degenerate. × 42.

Fig. 2.—Ovary of 17-day embryo showing the formation of the medulla. × 140.

Fig. 3.—Ovary of new-born mouse showing the structure of the ovary before the growth of the follicles (same fig. as fig. 5 in Plate 7 (4)). × 140.

Fig. 4.—Ovary of 13½-day embryo showing many of the germ-cells in synapsis. × 150.

We are indebted to Mr. F. J. Pittock for the photomicrographs reproduced in Plates 29–31. The drawings in Plate 28 were made with the aid of a camera lucida.

The Golgi Apparatus in the Cells of Tissue Cultures.

By R. J. Ludford, D.Sc., Ph.D., Lecturer in Cytology, University College, London.

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(From the Laboratories of the Imperial Cancer Research Fund.)

[Plates 32, 33.]

Introduction.

Although the Golgi apparatus can be seen in the cells of all tissues prepared by the appropriate cytological technique (9), yet up to the present nobody has succeeded in demonstrating a typical Golgi apparatus in the cells of tissue cultures. This is all the more remarkable since the Golgi apparatus can readily be seen in certain living cells (2, 12), and can be stained *intra vitam* by Janus green in others, while Brambell and Rau (13) have been able to trace the behaviour of the apparatus during spermatogenesis in *Cavia* by staining smears of the testis with Janus green, and fixing the cells by the iodine method of M. and W. H. Lewis (6).

Recently a paper has been published by Zweibaum and Elkner on the Golgi apparatus in tissue cultures (14). These investigators grew fragments of mesentery in both plasma and serum, and fixed their cultures in a variety of ways. They employed, amongst others, the osmic and silver impregnation methods. In only one case did they get a positive impregnation of the cells.

This result was obtained with the silver impregnation method of Da Fano. Among their illustrations they include a figure of a cell with a cluster of black granules grouped towards one side of the nucleus. This is regarded as one form of the apparatus in tissue cultures. Further, in the majority of the cells, which had become flattened out on the under surface of the cover-glass, numerous vacuoles were observed. Some of these were elongated and canalicular in form. Others were irregular. Since nothing but what are described as fat globules became impregnated by the osmic acid method, it was concluded that certain of the vacuolations represented a negative picture of the Golgi apparatus. Zweibaum and Elkner failed completely to trace any intermediate stages between the typical reticulate form of apparatus, and the juxta-nuclear group of granules in their one successful impregnation, and their only reason for regarding certain of the vacuoles as homologous with the Golgi apparatus seems to be that some of them happened to be elongated and tubular in shape.

Since it is quite obvious that the Golgi apparatus, if present at all, in the cells of tissue cultures must be in an altered form, it is essential before establishing homologies to trace the actual stages by which a typically reticulate Golgi apparatus becomes changed into an atypical form. That has been the aim of the research described in this paper.

Technique.

Cultures of the kidney of the rat embryo in Drew's saline medium containing embryo extract (3) were used in this research. I am indebted to Dr. A. H. Drew for the whole of the cultures which I have studied. In my early experiments, after the culture had been growing for two or three days, the fragment of tissue was picked off the cover-glass, and the cells which had grown out and were adhering to the surface of the glass were killed by fixing. Preparations were made by the Da Fano silver impregnation, and the Mann-Kopsch osmic acid impregnation methods. Since these methods failed to demonstrate anything like a typical Golgi apparatus in any of the cells, it was concluded that the methods were at fault. It seemed probable that the rapidity with which the cells were killed in the extended forms they assume in tissue cultures might explain the difficulty. Cultures were therefore allowed to remain without subculture, until many of the cells were obviously dead, and others were showing pathological changes. Such cultures were fixed and prepared in the same way as the others, but in none of the cells could a typical Golgi apparatus be seen.

The cultures in which I have succeeded in demonstrating a typical Golgi apparatus were fixed in Mann's corrosive osmic fluid, then washed in distilled water, and transferred to 2 per cent. osmic acid at 30° C. for two days. They were then examined under the microscope. Since the largest cells contained no reticulate structure, but simply black granules, the cultures were put back into 2 per cent. osmic acid, where they remained for three weeks. During this time they were regularly examined under the microscope, but no appreciable change was observed. There seems little doubt, therefore, that this three weeks' osmication of the cultures at room temperature could have been eliminated entirely, without affecting the final result. Since three weeks had been found by previous experiments to be the maximum time required to impregnate the Golgi apparatus at room temperature (7, 8), the osmication of the cultures was discontinued. They were then treated with water at 30° C. for one day to complete the reduction of the osmic acid, and were then brought up through the alcohols to absolute. The fragment of tissue—the explant—being brittle in absolute alcohol was readily removed with a needle. By keeping

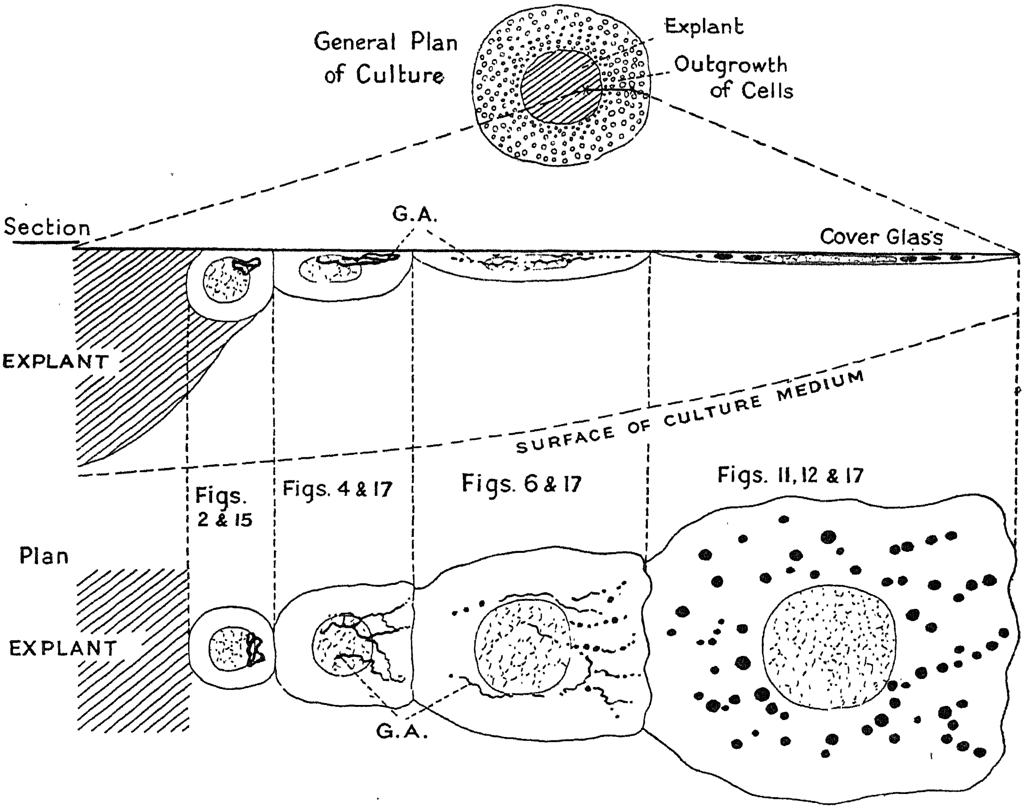


Fig. 1.—Diagram showing changes in the form of cells in tissue cultures due to their spreading out on the surface of the cover-glass. (GA—Golgi Apparatus.)

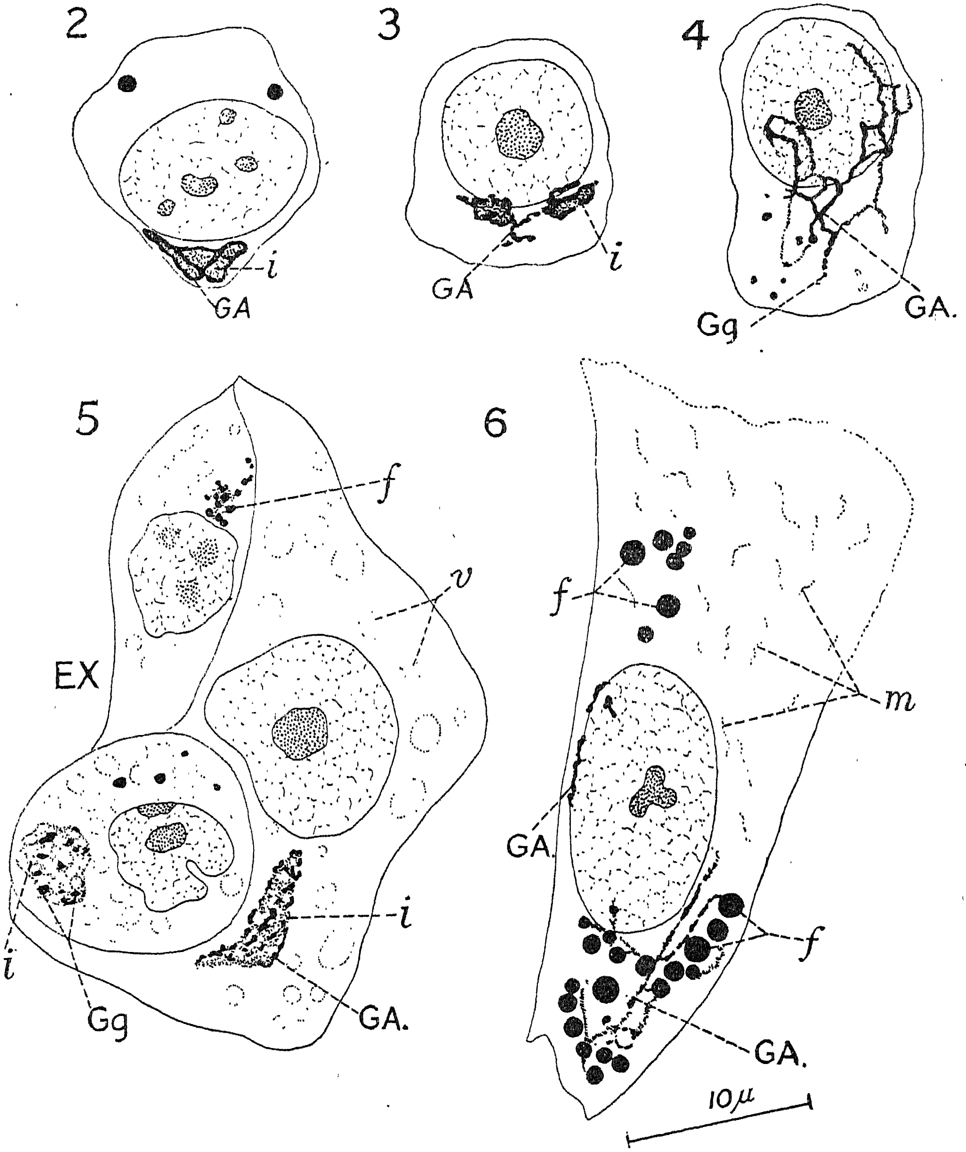
the explant on the cover-glass till this stage it is possible to retain its peripheral cells on the cover-glass. The innermost of these cells are little altered from the normal. In them a typically reticulate Golgi apparatus was impregnated by the technique employed. Cells further removed from the explant were more spread out. They showed the transformation which the Golgi apparatus undergoes as the cells become completely flattened out (see fig. 1). It was therefore possible by retaining the explant on the cover slip until a late stage to have sufficient cells in the preparation to trace the sequence of changes undergone by the Golgi apparatus of cells growing *in vitro*.

Staining was carried out after removal of the explants. The remaining cells were stained in neutral red for 10 minutes, and then differentiated in absolute alcohol, cleared in xylol, and mounted in balsam.

Variations in the Form of the Golgi Apparatus during the Migration of Cells from the Explant.

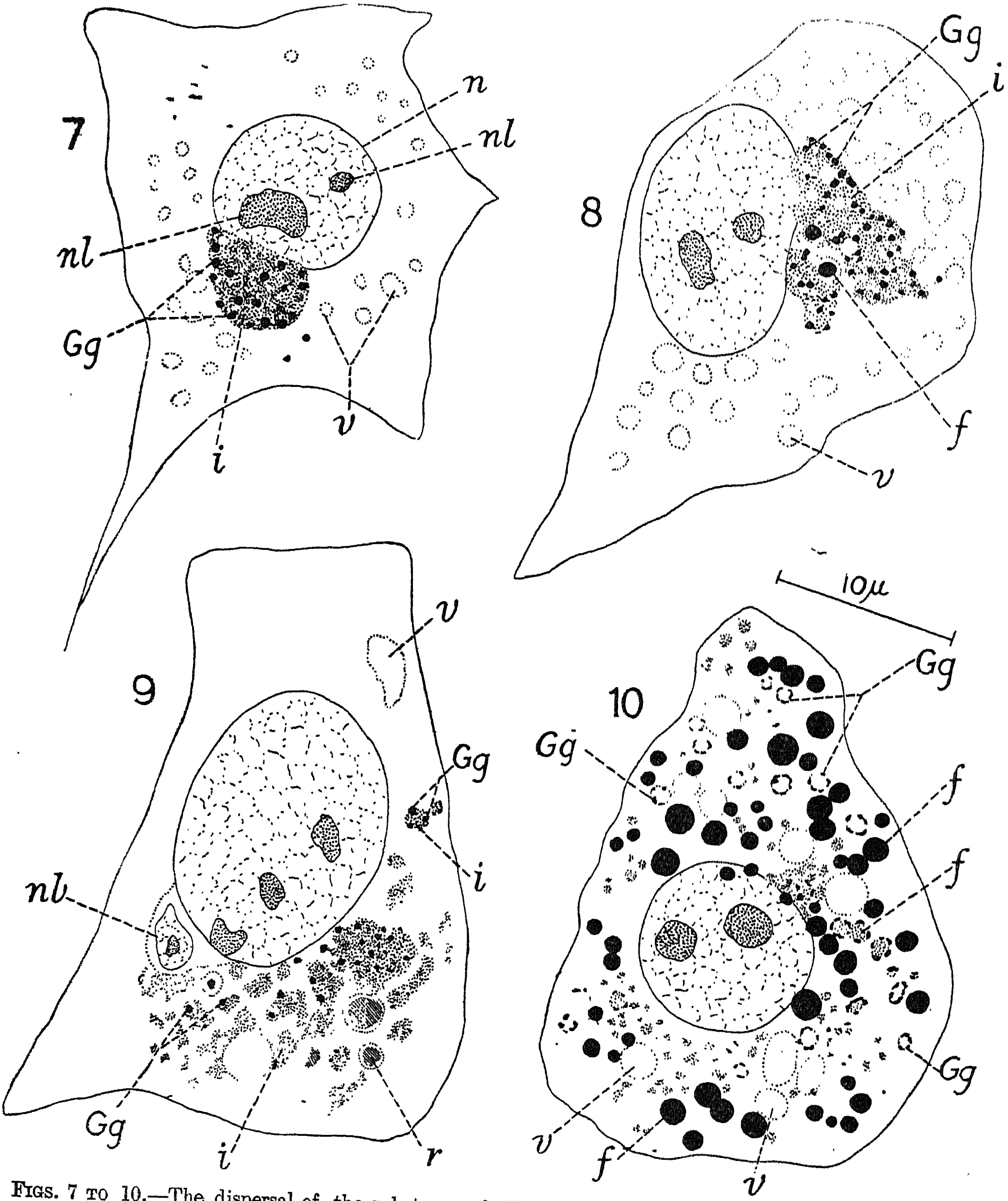
It has been found by experiment, that in order that cells may grow out from the explant into the culture medium, it is essential that some supporting structure should be present to which the cells may adhere. The supporting structure may be sterile fibres of cotton-wool, or spiders' web (4), but in most cultures the necessary surface for the cells to migrate upon is provided by the surface of the cover-glass. As the cells pass out from the fragment of tissue, they spread out upon the surface of the glass, until in the most extended condition they assume the form of a thin film of protoplasm adhering to the glass cover. This alteration in the form of the cells during growth *in vitro* is illustrated diagrammatically in fig. 1.

Cells around the immediate periphery of the explant are shown in the photomicrograph (Plate 32, fig. 15). In some of the cells the Golgi apparatus (GA) is seen as a dark compact mass at the side of the nucleus. A drawing of one of these cells is shown in fig. 2. The Golgi apparatus (GA) is here represented as an osmophil reticulum spread upon the less deeply impregnated mass of the sphere, or idiosomic substance, (*i*) fig. 3 shows a cell spread out slightly in a horizontal direction. The apparatus (GA) is in consequence stretched laterally. The cell represented in fig. 4, however, is drawn out longitudinally, and in consequence the apparatus (GA) is stretched in the same direction. It appears as a delicately drawn out network, breaking up at its extremities into granules (Gg). A more advanced stage in the process is shown in fig. 6. Here the apparatus (GA) is stretched to such an extent as to appear beaded, and in places there are large fatty or lipoidal droplets (*f*) arranged serially. Various stages in this drawing out of the apparatus are shown in the photomicrograph of fig. 17 (Plate 33).



FIGS. 2 TO 6.—The sequence of changes in the form of the Golgi Apparatus (GA) caused by flattening of the cells on the cover glass. (Ex, Explant; *f*, fatty or lipoidal droplets; GA, Golgi Apparatus; Gg, Golgi granules; *i*, idiosome; *m*, mitochondria; *v*, vacuole.)

Another form of this spreading out process is represented in figs. 5, and 7 to 10. Of the three cells shown in fig. 5, one has rounded off, and is filled with vacuoles.

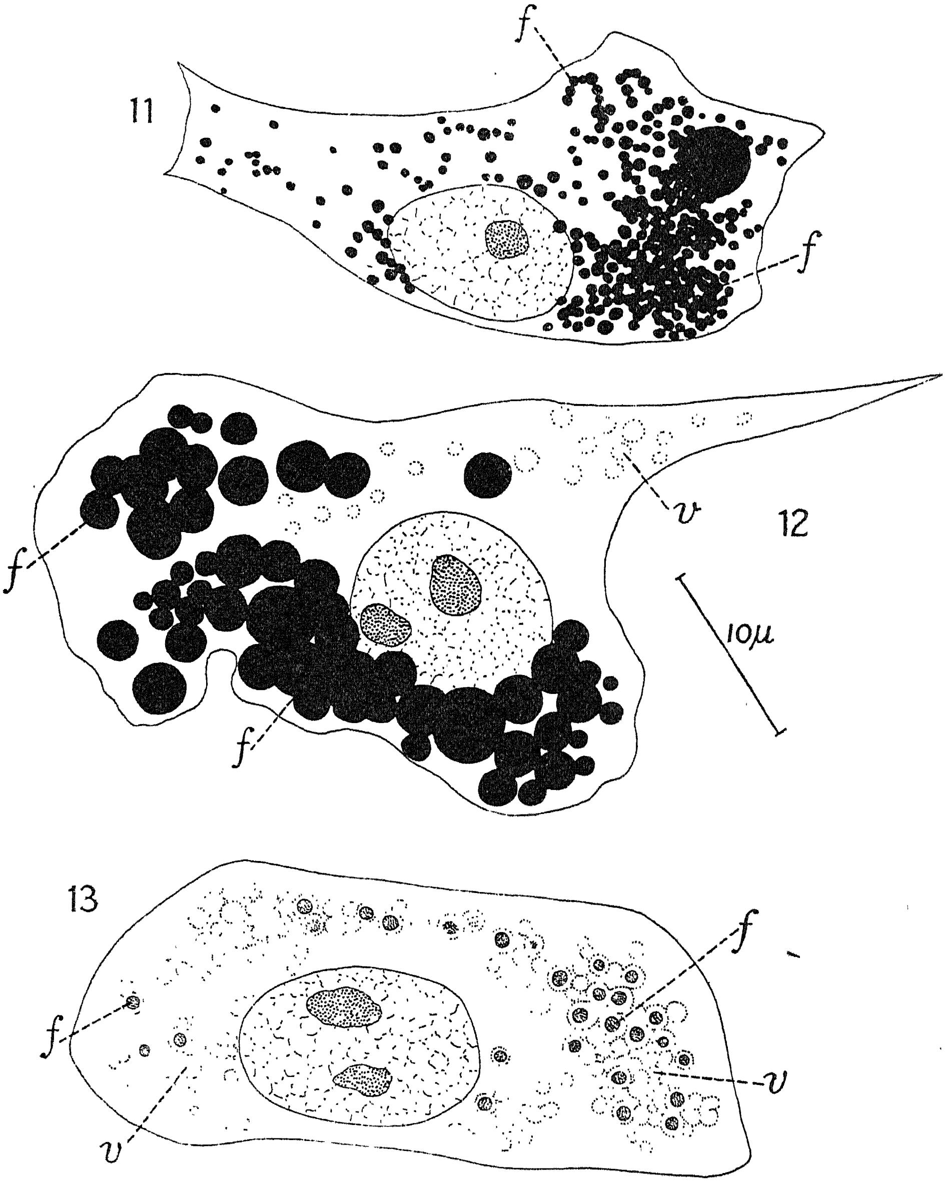


FIGS. 7 TO 10.—The dispersal of the substance of the Golgi Apparatus (GA) in the cytoplasm and the formation of fatty or lipoidal droplets. (*f*, fatty or lipoidal droplet; GA, Golgi Apparatus; Gg, Golgi granule; *i*, idiosomic substance; *n*, nucleus; *nb*, nuclear bud; *nl*, nucleolus; *v*, vacuole.)

Its nucleus is irregular in form and slightly pycnotic. It is quite clearly in a degenerate condition. The Golgi apparatus is represented by a number of granules (Gg) spread upon the less deeply impregnated sphere substance (*i*). The cell immediately above this one is in an abnormal condition. Fat droplets (*f*) appear to have been formed in relationship with its Golgi apparatus. The largest cell in the same figure is considerably stretched in the longitudinal direction. Its apparatus (GA) is drawn out in the same way, and assumes the form of rodlets and granules adherent to the idiosome (*i*). What is regarded as the sequence of changes in the spreading out of the Golgi apparatus when in this form is indicated in figs. 7 to 10. The cell shown in fig. 7 has the apparatus in the form of osmophil granules (Gg) spread upon the idiosome substance (*i*). Fig. 8 shows an early stage in its dispersal in the ground cytoplasm. The figures should be compared with the corresponding stages shown in the photomicrograph, fig. 16 (Plate 32). Fatty, or lipoidal globules (*f*) have already begun to form in the cell of fig. 8. In fig. 9 the dispersal of the Golgi material is more pronounced, while in fig. 10 it is almost complete. It appears that fat (*f*) is being formed in this latter cell in relationship with the Golgi granules (Gg), in a manner strikingly similar to that described during oögenesis of certain molluscs (5, 10), and during secretion in certain lipoidal glands (1, 11). It seems highly probable that finally the Golgi apparatus may become dispersed to such an extent that its individual particles may be below the limit of microscopic resolution.

The Origin of the Fatty or Lipoidal Droplets.

Certain investigators have attributed the origin of the fatty or lipoidal droplets of the cells of tissue cultures to mitochondria. M. and W. H. Lewis, however, state that they were unable to trace any relationship between fat and mitochondria, either in living cells stained *intra vitam*, or in fixed preparations (6). In cultures of rat kidney fixed in Flemming's fluid without acetic acid, and stained with iron hæmatoxylin, both mitochondria and fat globules are readily distinguishable ; but I have been unable to satisfy myself that there is any relation between the two. On the contrary, there is every indication from my preparations that part, at least, of the fat or lipoid of the cells is formed in relationship with the Golgi apparatus. If cultures, which have been fixed and osmicated by the method which I have employed, are treated with a 10 per cent. solution of turpentine in xylol for 24 hours all the fatty or lipoidal droplets appear as colourless vacuoles, on microscopic examination ; but in certain of the cells the Golgi material still remains in the form of black granules spread upon the idiosome. The cell shown in fig. 9 was bleached in this way, and the



Figs. 11 to 13.—The formation of fatty or lipoidal droplets (*f*) in the cells of tissue cultures. (*v*, vacuole.)

Golgi granules (Gg) are still clearly distinguishable. The cells with large fatty globules, after bleaching, contain no trace of osmophil granules. The study of such cells as those of fig. 10 and fig. 17 (Plate 33) has thus led me to conclude that in the cultures I have studied fat is formed in relationship with, or by the transformation of, Golgi bodies.

The cell shown in fig. 11, contains a considerable number of fatty globules (*f*), most of which are small in size. In several places it will be observed that these globules show a distinct serial arrangement as though they have been formed by the transformation of threads, or filaments, as shown in the cells of figs. 6 and 17 (Plate 33). One could also conceive of the globules (*f*) of fig. 12 arising by the enlargement of smaller granules, such as those of fig. 6. In other instances the fatty droplets originate as isolated intensely blackened granules of a very minute size. In such cases I consider it probable that they are formed in relationship with minute scattered particles, arising from the complete dispersal of the substance of the Golgi apparatus. There does, however, seem to be another method of fat formation. The cell shown in fig. 13 is extensively vacuolated. In certain of the vacuoles (*v*) are spherical droplets (*f*) which reduce osmic acid to varying degrees. Consequently, some of the globules are very faint, others are dark, while cells are found in which the fatty globules are intensely black. This method of fat

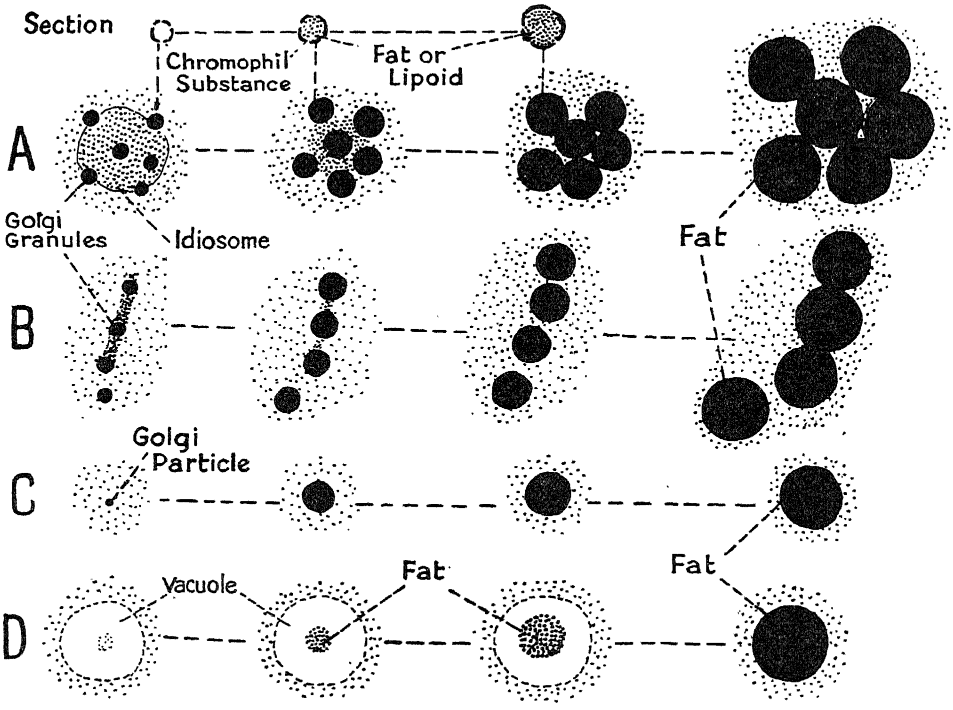


Fig. 14.—Diagram showing the ways in which fat is believed to be formed in the cells of tissue cultures.

formation differs from the other cases described in that the fatty droplets are first visible as faintly impregnated bodies of an appreciable size, within a vacuole. My interpretation of these methods of fat formation is represented diagrammatically in fig. 14.

Discussion.

The principal feature of this communication is the demonstration that with the spreading out of cells in tissue cultures the Golgi apparatus becomes stretched to such an extent that it is broken up into fragments which are scattered throughout the cytoplasm. Incidentally this raises anew the problem of the nature of the Golgi apparatus. Among recent workers Gatenby and Bowen have emphasised the dual nature of the Golgi complex. They point out that there are two different substances, as indicated by their relative capacities for reducing osmic acid. There is the chromophil substance, which appears deeply black in successful preparations, and a chromophobe material, which is only slightly blackened. The latter has been identified with the sphere, or the idiosomic substance. The two categories of substances are clearly distinguishable in figs. 2, 3, 5, and 7 to 9. In figs. 2 and 3 the chromophil Golgi substance (GA) appears to be arranged in the form of a network on the chromophobe material, which I regard as the sphere or idiosome. This latter substance is no longer distinguishable when the chromophil threads become drawn out as in figs. 4 and 6. It is impossible, from my preparations, to determine the fate of the chromophobe substance. Exactly the same problem has arisen previously in connection with the changes in the form of the apparatus during hypertrophy of the fibroblasts of tar tumours (11). It is possible in both these cases that the idiosomic substance is either spread in a thin film over a chromophil core, or it may be that the two are combined in such a way that the two substances are not distinguishable optically.

The process of spreading out of the Golgi apparatus shown in figs. 2 to 4 and fig. 6 is quite different morphologically from that shown in figs. 5, and 7 to 10. I am inclined to regard the former cells as epithelial in character, and the latter as fibroblasts; but a definite diagnosis is impossible. In the largest cell of fig. 5, we have the stage in which the filaments of the apparatus (GA) are being stretched and becoming attenuated to such an extent that fragmentation is occurring. This would lead finally to a condition such as that seen in fig. 7, where the osmophil fragments (Gg) are scattered upon the less deeply impregnated idiosomic substance (*i*). Further flattening of the cell would then result in the spreading out of the Golgi substance, as shown in figs. 8 and 16

(Plate 32). Eventually, this may result in a complete dispersal of the Golgi substance, stages of which are seen in figs. 9 and 10. Concurrently with this process, there is the formation of fat droplets (*f*) seen in fig. 10. In spite of differences then, the essential features of both these processes is the same. They both result ultimately in the scattering throughout the cytoplasm of the Golgi substance in the form of granules.

Summary.

The Golgi apparatus in the cells of tissue cultures undergoes a change in form with the spreading out of the cells on the surface of the cover-glass (fig. 1). As some cells spread out the Golgi apparatus is stretched until it fragments, and its individual particles become dispersed in the cytoplasm (figs. 2 to 4, 6 and 17 (Plate 33)). In other cells the osmophil substance of the Golgi apparatus becomes spread in the form of rodlets and granules upon a less deeply impregnated material (the idiosome, or sphere substance). Both substances become scattered together in the cytoplasm as the cells flatten out (figs. 5, 7 to 10 and 16 (Plate 32)). It seems probable that they may eventually become dispersed to such an extent that the individual particles are below the limit of microscopic resolution. Certain of the fatty, or lipoidal globules, in the cells of tissue cultures are considered to arise in relationship with the Golgi apparatus (fig. 14).

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DESCRIPTION OF PLATES.

PLATE 32.

FIG. 15.—A portion of the immediate periphery of the explant, showing the form of the cells before becoming flattened out on the cover glass. (Ex, explant; *f*, fatty, or lipoidal droplet; GA, Golgi Apparatus.)

FIG. 16.—Two cells moving away from the explant, showing the Golgi granules scattered over the surface of the idiosomic substance. (G.A., Golgi granules on idiosomic substance.)

PLATE 33.

FIG. 17.—The breaking up of the filaments of the Golgi Apparatus, due to the spreading of the cells on the surface of the cover glass, and fat droplets being formed in relationship with the resulting fragments.

I am indebted to Mr. F. J. Pittock of the Anatomy Department of University College for the above photomicrographs.

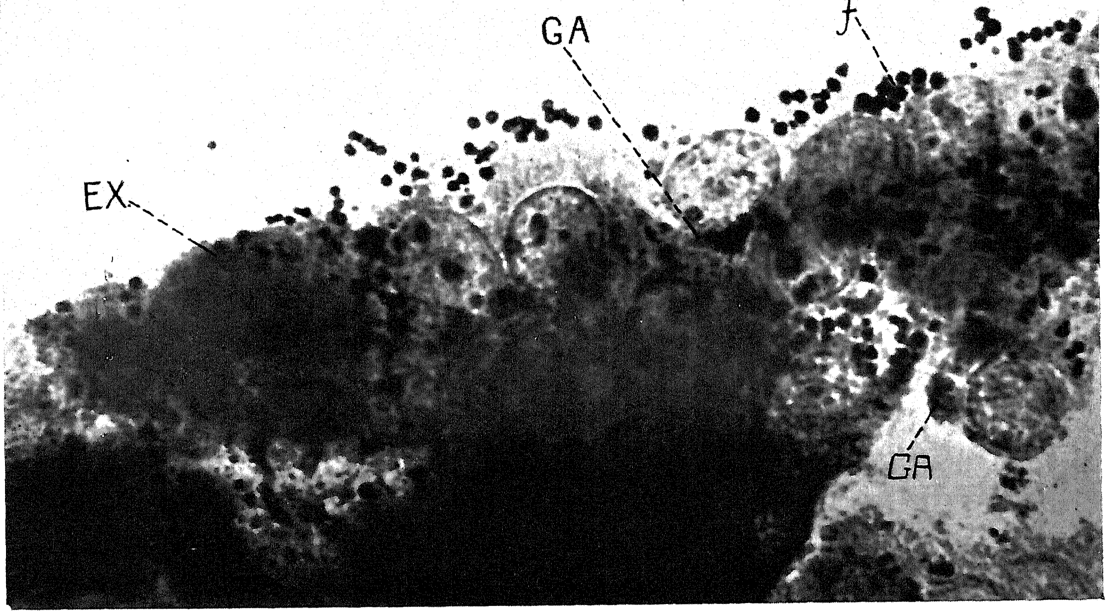

FIG. 15.

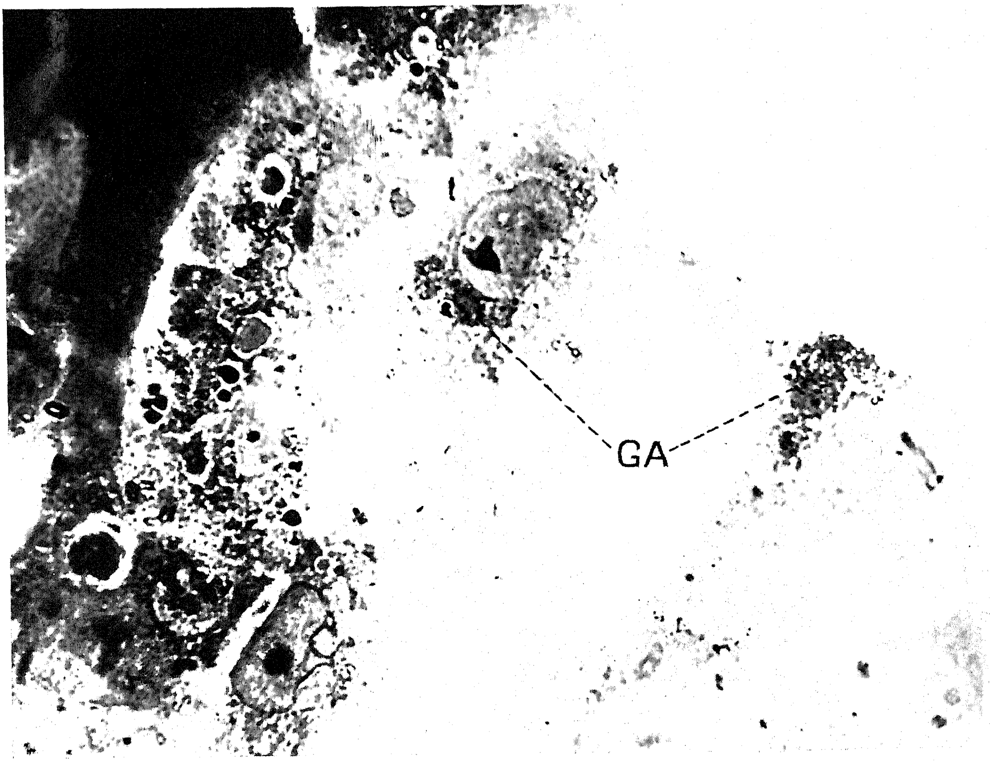

FIG. 16.

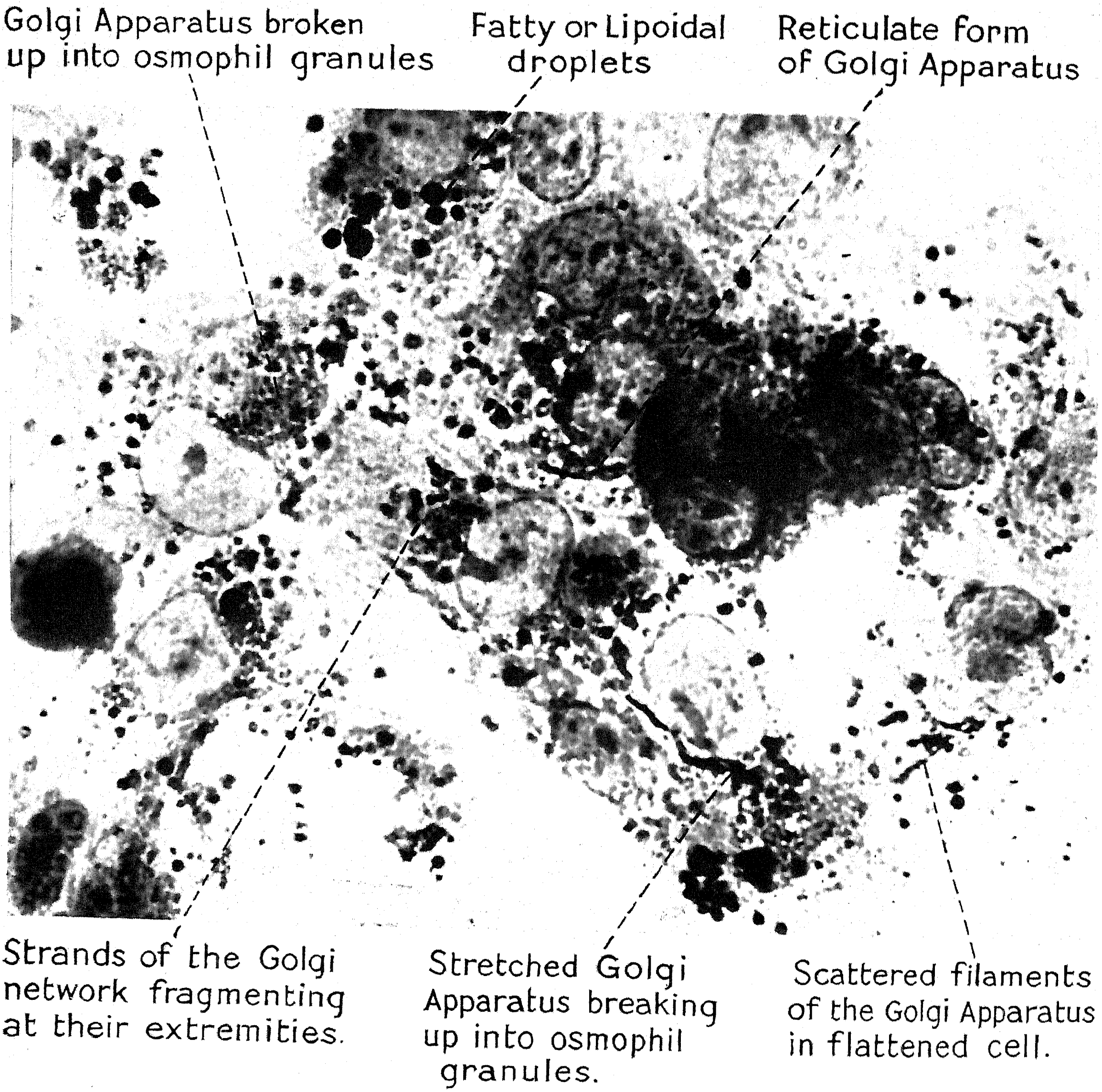
Golgi Apparatus broken up into osmophil granules
Fatty or Lipoidal droplets
Reticulate form of Golgi Apparatus
Strands of the Golgi network fragmenting at their extremities.
Stretched Golgi Apparatus breaking up into osmophil granules.
Scattered filaments of the Golgi Apparatus in flattened cell.

Fig. 17.

On the Occurrence of the Œstrous Cycle after X-Ray Sterilisation. Part III.—*The Periodicity of Œstrus after Sterilisation of the Adult.*

By A. S. Parkes, Beit Memorial Research Fellow.

(Communicated by Prof. J. P. Hill, F.R.S.—Received March 2, 1927.)

(From the Department of Physiology and Biochemistry,
University College, London.)

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I. *Introduction.*

In Parts I and II (2, 3, 10, 11) of these series it has been shown that the destruction of the Graafian follicles in the young female mouse by exposure to X-rays does not inhibit the appearance of œstrous symptoms when the animals subsequently become mature. The conclusions arrived at were briefly as follows :—

(*a*) After irradiation of the young animal the follicles disintegrate and are re-absorbed, this process being accompanied in its later stages by the proliferation of new tissue from the germinal epithelium.

(*b*) When the animal becomes adult the ovary contains neither organised follicles nor corpora lutea vera, and consists largely of post-irradiation proliferation of the germinal epithelium. In spite of this, however, the cyclic phenomena of œstrus still appear.

(*c*) Owing to the scarcity of original ovarian tissue in these sterilised ovaries, it was tentatively suggested that post-irradiation tissue might be responsible for the production of the hormone (œstrin) required for the causation of œstrus.

(*d*) These results further indicate that the synchronisation of œstrus and follicle maturation in the normal animal is brought about, not by the maturing follicles being the essential organ of elaboration of œstrin, but by both follicle maturation and œstrus being the expression of periodic ovarian activity.

(*e*) How this periodic activity was produced in an organ possessing no cyclic structures was not certain, but the periodic attainment of a "threshold" concentration of œstrin was suggested as a possible explanation.

The fact that in animals irradiated at or before three weeks old the tissue of post-irradiation epithelial origin appeared to be the probable source of œstrin in the sterilised ovary made comparison with the normal animal difficult, and furthermore, since this new tissue does not appear in quantity for some weeks, it seemed possible that some anœstrous gap might be found in the irradiated adult between the beginning of disintegration of the follicles and the appearance of post-irradiation tissue.

The experiments recorded in the present paper were begun in order to ascertain what effect obliteration of the follicles in the mature animal had upon the continuance of the œstrous cycle, and if the cycle persisted after irradiation, to ascertain in particular whether any hiatus occurred during the period when the follicles were disintegrating.

Every animal was previously examined in order that none showing gross abnormality in the cycle should be irradiated. All animals showing any such abnormalities were discarded. Most of the animals were not mated at any period of the experiment, and were left for some considerable time after irradiation. Some few of the animals, however, were mated after irradiation in order that the duration of fertility after exposure to a sterility dose might be ascertained. In addition, a series of animals were killed at graduated intervals after irradiation in order to provide material for the histological study of the post-irradiation changes.

It may be emphasised here that the question is no longer as to whether or not œstrus occurs after sterilisation. It has already been shown in Part I that the actual œstrous period of the sterilised animal has all the histological and physiological features (excepting ovulation) of that of the normal animal. The question is now : Is the periodicity of œstrus the same after sterilisation ? In the animals previously described, however, the data were insufficient to determine whether the interval between successive œstrous periods, *i.e.*, the length of the œstrous cycle, was also indistinguishable from the normal, and it is to this problem that the present physiological and statistical paper is devoted.

During the time these experiments were being carried out, the mouse colony was maintained with the aid of a grant from the Government Grants Committee of the Royal Society.

I wish to express my continued obligation to Prof. J. P. Hill, F.R.S., and Dr. F. H. A. Marshall, F.R.S., for their interest in the work. In addition, I am greatly indebted to Dr. E. S. Pearson for assistance with the statistical treatment of the results—the first attempt, so far as I know, to ascertain by biometric methods the effect of treatment on the length of the œstrous cycle.

As a result of the appearance of Part I of this series, and while the experiments described here were in progress, I received a copy of the MS. of a paper by Dr. J. Schubert, of Berlin, who had also found that œstrus occurred after X-ray sterilisation. The MS. arrived too late for adequate mention in Part II of this series, and I should like to take this additional opportunity of expressing my appreciation of Dr. Schubert's courtesy in allowing me to read the profusely illustrated MS. of his unpublished paper. Although measured in different units, Dr. Schubert appears to have used a much greater dosage than was the case in the experiments described in this and previous papers. He was able to show, as with the mice discussed in Part I, that the excision of the sterilised ovary immediately stopped the cycle, showing conclusively that the actual tissue removed and examined histologically was responsible for the occurrence of œstrus. He also claims that a second exposure to X-rays has a similar effect, but his material seems to be insufficient to demonstrate this definitely.

II. *Material and Methods.*

Sixty-six animals in all were examined with the object of selecting for irradiation animals showing a very regular cycle. Of these 66, 45 were finally irradiated, of which 27 were used for prolonged examination of the cycle without mating, and the remaining 18 were mated or used for early histological work. Of the 27 used for the œstrous cycle work, 7 were found on autopsy to be incompletely sterilised. In no case was more than an odd follicle or two discernible, but as our criterion of sterility is complete absence of germ cells, these animals had to be discarded from the main series. A number of these incompletely sterilised animals, however, showed features of interest, and will be discussed later. The number of normal cycles observed before treatment varied in the different animals, but in the case of the main series not less than three were observed in any case, while in some animals as many as ten pre-irradiation cycles were observed. In the vast majority of cases irradiation took place during diœstrus, only two of the main series of animals being irradiated during œstrus. This uniformity seemed desirable from an experimental point of view, especially as the effect on the cycle during which irradiation took place will be considered separately, but it was actually found that no histological or physiological difference could subsequently be detected, whatever the stage at which irradiation took place.

The time during which examinations were made after irradiation varied in the different animals from 2 days to 73 days, but the great majority were examined for not less than two months. Data are thus available for (*a*) cycles before irradiation, (*b*) the actual cycle during which irradiation took place, (*c*) cycles during the period of follicular degeneration, (*d*) cycles after the disappearance of the follicles as organised structures.

Dosage of X-Rays.—Eighty minutes of the standard intensity (10) amounting to approximately $\frac{1}{4}$B tint on Levi's pastilles, was known to sterilise young animals almost invariably. In adults this exposure was found to be less uniformly effective, but since it was desired for general reasons to keep the dosage as low as possible, the amount was not increased for the animals under discussion. The ovaries of every animal had to be examined histologically however, and it was easy to discover animals which retained any follicles. It was found that the disappearance of the follicles as such takes as long as five to six weeks, but of animals kept this time or longer after irradiation more than two-thirds were sterilised by the dosage used.

Detection of Œstrus.—The animals were in all cases examined for the detection of the œstrous cycle by the vaginal smear technique, first applied to the mouse by Allen (1). This technique has been described in Part I and need not be discussed again. In cases where the animals were mated after irradiation, the occurrence of copulation was, of course, used as corroboration of the smear diagnosis.

As in previous work, one cycle has been considered as running from diœstrus (including meta-œstrus) to œstrus (including pro-œstrus), the former period comprising roughly the time during which leucocytes are present in the vaginal smear, and the latter the time during which the smear consists solely of cornified or nucleated epithelium.

Statistical Treatment.—In order to compare the relative length of the cycles, and of their constituent phases, it is essential to arrive at some method whereby the validity of average values may be ascertained. In a case such as the œstrous cycle of the mouse, where even the values given by the normal animal are subject to much variation, the validity of any given mean figure clearly depends on the number of cycles observed. The obvious method, of course, would be to examine the frequency distribution of the length of cycle of the normal animal before irradiation, and to calculate σ, the deviation standard of the mean, and the probable error. The values given by the normal animal could then be compared with those given by the irradiated animal.

Two possible objections, however, exist to this method. In the first place, a number of cycles were observed in each animal, so that any tendency for an individual to have an idiosyncrasy in its cycle would render the material not a random sample. In the second place, the distribution about the mean is not symmetrical. Since, however, the asymmetry is not very marked, this second objection is scarcely serious enough to invalidate the method.

To consider the question of whether individual idiosyncrasy in length of cycle occurs, and if so, whether it persists after irradiation, Dr. Pearson has examined the data by forming six correlation tables in which the two variables x and y are as follows :—

(1) $x =$ a cycle before irradiation, $y =$ another cycle of same mouse before irradiation.

(2) $x =$ a cycle after irradiation, $y =$ another cycle of same mouse after irradiation.

(3) $x =$ a cycle after irradiation, $y =$ another cycle of same mouse before irradiation.

(4) (5) and (6) x and y corresponding values for the œstrous periods.

The pairs x and y were taken in every possible manner, and of the resulting tables only one showed a coefficient of correlation which was even barely significant. From this analysis Dr. Pearson concludes that the material available provides no evidence of individual peculiarities in length of cycles or œstrous periods.* The different intervals appear to be distributed among the mice in a perfectly random manner, so that a comparison of the frequency distributions of the cycles to be compared should provide a satisfactory method of dealing with the material.

---

* This in itself is an observation of some importance, and it is hoped to go into the point later. As regards the present data, however, it must be remembered that animals which showed a markedly abnormal cycle, and might, therefore, be supposed to show individual peculiarity in mean length most strongly, were weeded out before irradiation. In short, the normal material dealt with here provides a control for these experiments only.

The various values given by the frequency distributions have been calculated from the following formulæ :—

Standard deviation of mean $= \frac{\sigma}{\sqrt{N}}$, where N is number of cycles available.

Probable error of mean $= 0{\cdot}6745 \frac{\sigma}{\sqrt{N}}$.

Probable error of difference of two means :—

$$0{\cdot}6745 \sqrt{\left(\frac{\sigma_1}{\sqrt{N_1}}\right)^2 + \left(\frac{\sigma_2}{\sqrt{N_2}}\right)^2}.$$

The number of 24-hourly examinations at which one phase was observed appears, of course, in the tables as the number of days for which the phase was observed. Actually, of course, detection at one examination means that the phase lasted $>0 - <2$ days ; detection at two examinations means a duration of $>1 - <3$ days, and so on. The mean value for length of cycle or phase calculated from the tables is, therefore, considered as a true mean.

III. *Histological Effects of Irradiation of the Adult.*

The histology of the ovaries of the animals described in this paper is being dealt with elsewhere, in collaboration with Dr. Brambell, and this aspect of the work will not be dealt with here in any detail. It may, however, be pointed out that considerable difference exists between the post-irradiation changes in the ovary of the adult and the corresponding changes in the ovary of the young animal. In the latter case irradiation is followed by complete destruction of the follicles, and subsequently by proliferation of the germinal epithelium until new tissue comes to form most of the sterilised ovary. Pre-existing tissue, therefore, forms but little of the sterilised ovary of the young animal. Corpora lutea atretica are, however, often found.

In the case of the ovary of the animal sterilised after puberty, however, no proliferation of the germinal epithelium has been observed, and the tissues of the sterilised ovary of the adult appear to be formed entirely from the existing cortical tissues, *i.e.*, follicular and interfollicular material.

It should, however, be emphasised that one fundamental similarity exists between the ovaries of animals sterilised both before and after puberty. In neither case has the most careful histological examination revealed any cyclic histological change which might form a basis for cyclic endocrine activity. In the animal sterilised when adult, both corpora lutea vera (old) and corpora lutea atretica are found, but these appear to be histologically "permanent" and functionless. In the circumstances it must be supposed that no histological cycle exists in the sterilised ovary at all comparable with the alternating maturation of Graafian follicles and corpora lutea which is found in the normal animal. This absence of histological periodicity in the ovary associated with the normal production of the cyclic changes in the accessory organs presents the chief problem arising from the fact that the œstrous cycle persists after sterilisation. Fuller discussion of this point is reserved to Section VI of this paper.

Finally, it may be mentioned that the period during which sterilisation changes are taking place appears to last for some 5 to 6 weeks, and some semblance of follicles may be found up to this time in animals which will ultimately become quite sterile. This point is of importance in considering the nature of the post-irradiation œstrous cycle.

IV. *The Effect of Sterilisation on the Œstrous Cycle of the Unmated Female.*

(*a*) *Abnormal Cases.*—Since the animals as a whole showed a perfectly satisfactory cycle after irradiation, both during the time when the follicles were undergoing destruction as organised units and afterwards, it is unnecessary to give more description of most individual cases than is contained in the tables which follow. At the same time, however, a few abnormalities were found which are not shown by the cycle formulæ. Since only complete cycles (meta-œstrus to œstrus) are included in these formulæ, incomplete portions of cycles observed immediately before autopsy are not represented, and in a few cases the period between the end of the last cycle and autopsy observed was abnormal in one way or another. Cases of unduly prolonged absence of œstrus previous to autopsy are given in Table I.

Table I.

Number of animal.	Time of autopsy after irradiation. Days	Nature of peculiarity.	Notes.
AX 9	64	Absence of œstrus for 19 days previous to autopsy	Viscera very fat.
AX 23	66	Absence of œstrus for 31 days previous to autopsy	—
AX 49	55	Absence of œstrus for 18 days previous to autopsy	Animal in bad condition.
AX 51	55	Absence of œstrus for 23 days previous to autopsy	—

The fatness of AX 9 and the bad condition of AX 49 may well explain the absence of œstrus in these cases. Only one other obvious irregularity was found which is not brought out by the cycle formulæ. In AX 46 the last 29 days of the period of observation were occupied by a diœstrous interval of 18 days, which was followed by incomplete œstrous cornification lasting for 11 days. In no one of these abnormal cases could any definite histological peculiarity of the ovary be found in correlation, but at the same time these few abnormalities are no more curious than anomalies occasionally found in the untreated animal, and they are quite insufficient to affect the generalisation that the cycle persists after sterilisation.

(*b*) *The Cycle before Sterilisation.*—Before considering in detail the effect of irradiation on the cycle, it will be convenient to deal with the normal cycles exhibited by the same animals before treatment. The following table shows the number of pre-irradiation cycles and their length in days (calculated as described above) in each of the 20 animals which were afterwards completely sterilised :—

Table II.—Length of Pre-Irradiation Cycles.

Number of animal.	Number of cycles.	Lengths of cycles. Days.	Average.	Total days.
AX 1	3	12–6–4	7·3	22
AX 2	5	5–4–4–5–10	5·6	28
AX 3	5	6–6–4–7–5	5·6	28
AX 7	4	9–6–6–4	6·2	25
AX 9	4	11–6–5–5	6·7	27
AX 16	9	9–5–12–8–4–4–12–4–3	6·8	61
AX 17	10	4–5–6–5–14–4–5–6–5–5	5·9	59
AX 19	8	11–5–12–6–7–5–4–6	7·0	56
AX 20	7	7–7–11–6–7–8–6	7·4	52
AX 23	8	5–5–8–18–4–7–7–4	7·3	58
AX 33	9	6–7–5–5–3–5–5–5–6	5·2	47
AX 37	8	5–5–3–4–5–6–5–5	4·8	38
AX 41	5	5–5–7–5–5	5·4	27
AX 44	7	6–4–3–5–3–7–4	4·6	32
AX 45	7	7–5–4–5–5–5–5	5·1	36
AX 46	3	5–4–12	7·0	21
AX 47	4	5–5–7–5	5·5	22
AX 49	3	6–5–7	6·0	18
AX 51	3	6–7–4	5·7	17
AX 53	4	4–6–5–5	5·0	20
Total	116		5·98	694

In these 20 sterilised animals, therefore, 116 œstrous cycles averaging 5·98 days in length were observed. The average length of cycle for individual animals was fairly constant, varying only from 4·6 days to 7·3 days. The frequency distribution of length of cycle for these 116 cycles is as follows :—

Table III.—Frequency Distribution of Length of Pre-Irradiation Cycles.

Length of cycle in days.	Number of cycles.	Total days occupied.
3	5	15
4	20	80
5	42	210
6	19	114
7	14	98
8	3	24
9	2	18
10	1	10
11	3	33
12	5	60
13	0	—
14	1	14
15	0	—
16	0	—
17	0	—
18	1	18
Total	116	694

For this frequency distribution $\sigma = 2 \cdot 44$ and the s.d. of the mean $= \frac{2 \cdot 44}{\sqrt{116}} = 0 \cdot 227$. The mean length of the cycle is thus 5·98 days, with an s.d. of 0·227, the probable error being 0·153 days. This figure, therefore, gives a standard with which the experimental cycles may be compared.

Analysing the results according to the constituent parts of the cycles it has also been possible to obtain normal values for the length of diœstrus and œstrus (using these terms as explained in Section II).

The following table gives the lengths of diœstrus in the various animals :—

Table IV.—Length of Pre-Irradiation Diœstrous Periods.

Number of animal.	Number of periods.	Lengths of diœstrus. Days.	Average.	Total days.
AX 1	3	10–3–2	5·0	15
AX 2	5	2–2–3–3–8	3·6	18
AX 3	5	2–3–2–3–3	2·6	13
AX 7	4	7–3–4–2	4·0	16
AX 9	4	9–4–4–3	5·0	20
AX 16	9	4–2–10–5–2–2–8–2–2	4·1	37
AX 17	10	3–3–4–3–11–2–3–4–2–3	3·8	38
AX 19	8	8–2–6–4–4–3–2–3	4·0	32
AX 20	7	4–4–9–4–4–5–4	4·9	34
AX 23	8	3–3–5–15–2–5–5–3	5·1	41
AX 33	9	4–5–3–3–2–3–3–3–4	3·3	30
AX 37	8	2–2–2–2–3–4–2–3	2·5	20
AX 41	5	3–3–2–3–2	2·6	13
AX 44	7	3–2–2–3–2–4–2	2·6	18
AX 45	7	4–2–3–3–3–3–2	2·9	20
AX 46	3	2–2–9	4·3	13
AX 47	4	3–2–4–3	3·0	12
AX 49	3	2–3–4	3·0	9
AX 51	3	4–5–2	3·7	11
AX 53	4	2–4–3–3	3·0	12
Total	116		3·64	422

The 116 diœstrous periods thus had an average length of 3·64 days. The frequency distribution of the length of diœstrous period is as follows :—

Table V.—Frequency Distribution of Length of Pre-Irradiation Diœstrous Periods.

Length in days.	Number of periods.	Total days occupied.
2	36	72
3	39	117
4	22	88
5	7	35
6	1	6
7	1	7
8	3	24
9	3	27
10	2	20
11	1	11
12	0	—
13	0	—
14	0	—
15	1	15
Total	116	422

For this frequency distribution $\sigma = 1 \cdot 67$, and the mean of 3·64 days therefore has an s.d. of $\frac{0 \cdot 167}{\sqrt{116}} = 0 \cdot 155$ and a probable error of 0·104.

The lengths of the pre-treatment œstrous periods are given below in Table VI.

Table VI.—Length of Pre-Irradiation Œstrous Periods.

Number of animal.	Number of periods.	Lengths of œstrus. Days.	Average.	Total days.
AX 1	3	2–3–2	2·3	7
AX 2	5	3–2–1–2–2	2·0	10
AX 3	5	4–3–2–4–2	3·0	15
AX 7	4	2–3–2–2	2·2	9
AX 9	4	2–2–1–2	1·7	7
AX 16	9	5–3–2–3–2–2–4–2–1	2·7	24
AX 17	10	1–2–2–2–3–2–2–2–3–2	2;1	21
AX 19	8	3–3–6–2–3–2–2–3	3·0	24
AX 20	7	3–3–2–2–3–3–2	2·6	18
AX 23	8	2–2–3–3–2–2–2–1	2·1	17
AX 33	9	2–2–2–2–1–2–2–2–2	1·9	17
AX 37	8	3–3–1–2–2–2–3–2	2·2	18
AX 41	5	2–2–5–2–3	2·8	14
AX 44	7	3–2–1–2–1–3–2	2·0	14
AX 45	7	3–3–1–2–2–2–3	2·3	16
AX 46	3	3–2–3	2·7	8
AX 47	4	2–3–3–2	2·5	10
AX 49	3	4–2–3	3·0	9
AX 51	3	2–2–2	2·0	6
AX 53	4	2–2–2–2	2·0	8
Total	116		2·35	272

The average length of the œstrous period is thus 2·35 days. The frequency distribution of length of œstrous period is given in Table VII.

Table VII.—Frequency Distribution of Length of Pre-Irradiation Œstrous Periods.

Length in days.	Number of periods.	Total days occupied.
1	10	10
2	67	134
3	32	96
4	4	16
5	2	10
6	1	6
Total	116	272

For this frequency distribution $\sigma = 0\cdot817$ and the standard deviation of the mean $= \dfrac{0\cdot817}{\sqrt{116}} = 0\cdot0759$. The mean is, therefore, $2\cdot35 \pm 0\cdot0512$.

These results show what might be expected, *i.e.*, that measured in days the variability of the length of the short œstrous period in the normal mouse is much less than that of the longer diœstrous period. As regards these pre-irradiation cycles, however, it should be remembered that they cannot be supposed to represent a random sample for the whole normal mouse colony on account of the fact that, as noted above, they were observed in animals specially selected for the regularity and normality of their cycle. These pre-irradiation cycles do, however, represent an accurate control on those observed in the same animals following irradiation.

(*c*) *The Cycle during which Irradiation took Place.*—Where irradiation took place at the beginning of diœstrus, it might have been thought that any œstrus-inhibiting effect of irradiation would be observable at the following œstrous period. This appeared all the more likely as the initial effects of irradiation are observable histologically very soon after exposure. At the same time, however, it is necessary to remember the cases where pregnancy followed mating after irradiation, which shows that follicles may retain for a short while their power of ovulation even in animals which ultimately become sterile.

The following table gives for each of the 20 sterilised animals the total length of the cycle during which irradiation occurred, the length of its constituent parts, and the stage of the cycle at which irradiation took place.

Table VIII.—Data relating to Cycles in which Irradiation occurred.

Number of animal.	Length of cycle. Days.	Diœstrus.	Œstrus.	Irradiation took place.
AX 1	5	3	2	Last day diœstrus.
AX 2	11	9	2	2nd day diœstrus.
AX 3	12	3	9	Last day diœstrus.
AX 7	7	4	3	3rd day diœstrus.
AX 9	9	7	2	3rd day diœstrus.
AX 16	2	1	1	Last day of œstrus.
AX 17	6	3	3	Last day diœstrus.
AX 19	4	2	2	Last day diœstrus.
AX 20	6	4	2	2nd day diœstrus.
AX 23	7	5	2	2nd day diœstrus.
AX 33	9	6	3	3rd day diœstrus.
AX 37	8	6	2	1st day diœstrus.
AX 41	11	8	3	6th day diœstrus.
AX 44	9	7	2	3rd day diœstrus.
AX 45	8	5	3	2nd day diœstrus.
AX 46	10	4	6	4th day diœstrus.
AX 47	6	3	3	1st day diœstrus.
AX 49	6	2	4	2nd day œstrus.
AX 51	7	5	2	1st day diœstrus.
AX 53	9	5	4	3rd day diœstrus.
Total........	152	92	60	

For these 20 cycles the following values may be calculated:—

Mean total length of cycle	7·60 days
Mean length of diœstrus	4·60 ,,
Mean length of œstrus	3·0 ,,

Each of these values is higher than the corresponding one given by the pre-irradiation cycles, but it seems difficult to attach much importance to this fact. In the first place, it is impossible to compare the two sets of values accurately, owing to the data for the cycles during which irradiation took place being too meagre for the calculation of a reliable probable error, and any error calculated would be so large as to invalidate the difference from the normal. In the second place, the abnormality of one or two of the cycles could not have been due to the coincident irradiation owing to the fact that it took place at a late stage of the cycle. Of the 20 cycles, 15, those of AX 1, 7, 9, 16, 17, 19, 20, 23, 33, 37, 44, 45, 47, 51, 53, must be considered as being within the usual range, both as regards their total length and also as regards the actual and relative length of diœstrus and œstrus.

As regards the remainder the following remarks may be made:—In AX 2 and 3 irradiation took place early in the cycle, and the abnormal length of diœstrus in the first, and of œstrus in the second, might be connected with the exposure to X-rays. In AX 41, however, the length of diœstrus can hardly be attributed to the irradiation, which took place almost at the end of the period. The long œstrous period of the cycle in AX 46 might, however, be attributed to the irradiation, but the disproportion between the length of œstrus and diœstrus in AX 49 scarcely can be.

In three cases out of the twenty, therefore, disturbance of the cycle during which irradiation was performed might be attributed to the treatment; but as the apparent effect is different in each, it seems highly improbable that the disturbance is due to the irradiation. In any case it is quite clear that irradiation has no regular or definite effect on the occurrence of the next œstrous period and, therefore, that the beginning of atresia in the follicles is not incompatible with the production of œstrus.

(*d*) *The Cycle after Sterilisation.*—The data for the post-irradiation cycles of the 20 sterilised females are as follows:—

Table IX.—Length of Post-Irradiation Cycles.

Number of animal.	Number of cycles.	Lengths of cycles. Days.	Average.	Total days.
AX 1	6	7–16–14–8–10–14	11·5	69
AX 2	9	12–5–10–9–5–4–3–9–4	6·8	61
AX 3	9	10–5–2–6–8–6–13–6–5	6·8	61
AX 7	7	5–7–8–9–9–15–14	9·6	67
AX 9	4	8–11–14–8	10·2	41
AX 16	8	5–4–4–12–8–4–4–3	5·5	44
AX 17	6	5–6–4–5–6–6	5·3	32
AX 19	13	5–4–4–4–4–5–4–5–7–4–7–8–4	5·0	65
AX 20	11	13–5–4–5–4–6–4–3–5–9–5	5·7	63
AX 23	8	4–5–4–4–3–4–6–3	4·1	33
AX 33	8	8–6–6–9–2–6–3–10	6·3	50
AX 37	9	6–5–4–4–4–8–9–12–6	6·5	58
AX 41	9	5–4–4–6–10–4–6–4–8	5·7	51
AX 44	6	17–6–5–4–5–11	8·0	48
AX 45	10	6–6–5–8–9–3–5–8–5–5	6·0	60
AX 46	3	4–10–5	6·3	19
AX 47	5	6–7–5–5–8	6·2	31
AX 49	4	8–7–8–24	11·7	47
AX 51	5	4–6–5–4–6	5·0	25
AX 53	6	6–6–4–5–7–16	7·3	44
Total	146		6·64	969

The mean length of these 146 post-irradiation cycles is 6·64 days. The frequency distribution of the length of these cycles is as follows :—

Table X.—Frequency Distribution of Length of Post-Irradiation Cycles.

Length of cycle in days.	Number of cycles.	Total days occupied.
2	2	4
3	7	21
4	33	132
5	29	145
6	23	138
7	7	49
8	15	120
9	8	72
10	6	60
11	2	22
12	3	36
13	2	26
14	4	56
15	1	15
16	2	32
17	1	17
24	1	24
Total	146	969

For this series $\sigma = 4\cdot19$, and the standard deviation of the mean is $\frac{4\cdot19}{\sqrt{146}} = 0\cdot347$. The mean is therefore $6\cdot64 \pm 0\cdot234$.

The values for the diœstrous periods are given in Table XI.

Table XI.—Lengths of Post-Irradiation Diœstrous Periods.

Number of animal.	Number of periods.	Lengths of diœstrus. Days.	Average	Total days.
AX 1	6	4–14–12–3–5–12	8·3	50
AX 2	9	8–3–5–6–3–3–2–4–2	4·0	36
AX 3	9	6–2–1–2–4–3–11–2–3	3·8	34
AX 7	7	3–5–4–5–5–6–12	5·7	40
AX 9	4	5–5–11–4	6·2	25
AX 16	8	3–3–2–10–6–3–3–2	4·0	32
AX 17	6	3–4–2–3–4–4	3·3	20
AX 19	13	3–2–2–2–2–3–2–2–5–2–4–3–3	2·7	35
AX 20	11	11–2–2–3–2–3–2–2–4–7–3	3·7	41
AX 23	8	2–3–2–2–2–2–3–2	2·2	18
AX 33	8	3–3–3–5–1–4–2–8	3·6	29
AX 37	9	3–3–2–2–2–4–6–10–3	3·9	35
AX 41	9	4–2–2–3–7–2–3–2–5	3·3	30
AX 44	6	15–4–4–2–3–8	6·0	36
AX 45	10	3–4–3–4–6–2–3–5–2–2	3·4	34
AX 46	3	3–6–2	3·7	11
AX 47	5	3–3–3–3–4	3·2	16
AX 49	4	6–5–6–17	8·5	34
AX 51	5	3–4–3–3–2	3·0	15
AX 53	6	4–4–3–3–4–12	5·0	30
Total	146		4·12	601

The average length of these diœstrous periods is thus 4·12 days. The frequency distribution of the length of these periods is as follows :—

Table XII—Frequency Distribution of Length of Post-Irradiation Diœstrous Periods.

Length in days.	Number of periods.	Total days occupied.
1	2	2
2	40	80
3	44	132
4	22	88
5	12	60
6	9	54
7	2	14
8	3	24
9	0	—
10	2	20
11	3	33
12	4	48
14	1	14
15	1	15
17	1	17
Total	146	601

For this distribution $\sigma = 2\cdot87$, so that the s.d. of the mean $= \dfrac{2\cdot87}{\sqrt{146}} = 0\cdot237$. The mean is, therefore, $4\cdot12 \pm 0\cdot160$. The length of the œstrous periods of the post-irradiation cycles are given in Table XIII.

Table XIII.—Length of Post-Irradiation Œstrous Periods.

Number of animal.	Number of periods.	Lengths of œstrus. Days.	Average.	Total days.
AX 1	6	3–2–2–5–5–2	3·2	19
AX 2	9	4–2–5–3–2–1–1–5–2	2·8	25
AX 3	9	4–3–1–4–4–3–2–4–2	3·0	27
AX 7	7	2–2–4–4–4–9–2	3·9	27
AX 9	4	3–6–3–4	4·0	16
AX 16	8	2–1–2–2–2–1–1–1	1·5	12
AX 17	6	2–2–2–2–2–2	2·0	12
AX 19	13	2–2–2–2–2–2–2–3–2–2–3–5–1	2·3	30
AX 20	11	2–3–2–2–2–3–2–1–1–2–2	2·0	22
AX 23	8	2–2–2–2–1–2–3–1	1·9	15
AX 33	8	5–3–3–4–1–2–1–2	2·6	21
AX 37	9	3–2–2–2–2–4–3–2–3	2·6	23
AX 41	9	1–2–2–3–3–2–3–2–3	2·3	21
AX 44	6	2–2–1–2–2–3	2·0	12
AX 45	10	3–2–2–4–3–1–2–3–3–3	2·6	26
AX 46	3	1–4–3	2·7	8
AX 47	5	3–4–2–2–4	3·0	15
AX 49	4	2–2–2–7	3·2	13
AX 51	5	1–2–2–1–4	2·0	10
AX 53	6	2–2–1–2–3–4	2·3	14
Total........	146		2·52	368

The mean length of post-irradiation œstrus is therefore 2·52 days. The frequency distribution of length of post-irradiation œstrus is given in Table XIV.

Table XIV.—Frequency Distribution of Length of Post-Irradiation Œstrous Periods.

Length in days.	Number of periods.	Total days occupied.
1	21	21
2	70	140
3	29	87
4	17	68
5	6	30
6	1	6
7	1	7
9	1	9
Total	146	368

This distribution gives $\sigma = 1 \cdot 23$, and the mean length of œstrus (2·52 days), therefore, has an s.d. of $\frac{1 \cdot 23}{\sqrt{146}} = 0 \cdot 102$, and a p.e. of 0·0687.

(*e*) *Examination of Results. Average Length of Pre- and Post-Irradiation Cycles in Individual Animals.*—The average length of the cycles and their constituent parts before and after irradiation in each of the 20 individuals are compared below in Table XV.

These figures show no obvious difference in the length of cycle after irradiation. Of individual animals, 13 of the 20 had a slightly longer average length of cycle after irradiation, while the remaining 7 had a shorter average length of cycle. It is significant that the same 13 animals also had a greater average length of diœstrus. As regards œstrus, 4 animals had the same average length before and after irradiation. Of the others, 10 had a longer average and 6 a shorter average after irradiation. In only 3 cases, however, was the difference in average more than one day. From these figures, therefore, the effect, if any, of irradiation on the length of œstrus is even less marked than on the length of diœstrus.

Table XV.

No. of animal.	Whole cycle.			Diœstrus.			Œstrus.		
	Before irrad.	After irrad.	Difference after irrad.	Before irrad.	After irrad.	Difference after irrad.	Before irrad.	After irrad.	Difference after irrad.
AX 1	7·3	11·5	+4·2	5·0	8·3	+3·3	2·3	3·2	+0·9
AX 2	5·6	6·6	+1·0	3·6	4·0	+0·4	2·0	2·8	+0·8
AX 3	5·6	6·8	+1·2	2·6	3·8	+1·2	3·0	3·0	—
AX 7	6·2	9·6	+3·4	4·0	5·7	+1·7	2·2	3·9	+1·7
AX 9	6·7	10·2	+3·5	5·0	6·2	+1·2	1·7	4·0	+2·3
AX 16	6·8	5·5	−1·3	4·1	4·0	−0·1	2·7	1·5	−1·2
AX 17	5·9	5·3	−0·6	3·8	3·3	−0·5	2·1	2·0	−0·1
AX 19	7·0	5·0	−2·0	4·0	2·7	−1·3	3·0	2·3	−0·7
AX 20	7·4	5·7	−1·7	4·9	3·7	−1·2	2·6	2·0	−0·6
AX 23	7·3	4·1	−3·2	5·1	2·2	−2·9	2·1	1·9	−0·2
AX 33	5·2	6·3	+1·1	3·3	3·6	+0·3	1·9	2·6	+0·7
AX 37	4·8	6·5	+1·7	2·5	3·9	+1·4	2·2	2·6	+0·4
AX 41	5·4	5·7	+0·3	2·6	3·3	+0·7	2·8	2·3	−0·5
AX 44	4·6	8·0	+3·4	2·6	6·0	+3·4	2·0	2·0	—
AX 45	5·1	6·0	+0·9	2·9	3·4	+0·5	2·3	2·6	+0·3
AX 46	7·0	6·3	−0·7	4·3	3·7	−0·6	2·7	2·7	—
AX 47	5·5	6·2	+0·7	3·0	3·2	+0·2	2·5	3·0	+0·5
AX 49	6·0	11·7	+5·7	3·0	8·5	+5·5	3·0	3·2	+0·2
AX 51	5·7	5·0	−0·7	3·7	3·0	−0·7	2·0	2·0	—
AX 53	5·0	7·3	+2·3	3·0	5·0	+2·0	2·0	2·3	+0·3
Total	5·98	6·64	+0·66	3·64	4·12	+0·48	2·35	2·52	+0·17

Comparison of Mean Values.—The following table (XVI) summarises the difference in the means before and after irradiation, and shows the relation of the difference to their probable errors.

Table XVI.

—	Mean length.		Difference.	P.e. of difference.	Diff./p.e.
	Before irrad.	After irrad.			
Whole cycle	5·98 ± 0·153	6·64 ± 0·234	0·66	0·296	2·23
Diœstrus	3·64 ± 0·104	4·12 ± 0·160	0·48	0·190	2·52
Œstrus	2·35 ± 0·0512	2·52 ± 0·0687	0·17	0·0856	1·99

In view of their relations to their probable errors it seems impossible to attach any statistical significance to these differences, and they certainly have no physiological meaning. It must, therefore, be concluded that in the mouse the length of the œstrous cycle and of its constituent phases after irradiation is indistinguishable from that found before irradiation.

The Variability of Post-Irradiation Cycles.—It has been shown above that there is no significant difference between the mean length of the pre-irradiation and the mean length of the post-irradiation cycles. In the case of the post-irradiation cycles, however, the variability is somewhat greater, as is shown by the following comparison of standard deviations :—

	Pre-irradiation.	Post-irradiation.
Whole cycle	2·44	4·19
Diœstrus	1·67	2·87
Œstrus	0·817	1·23

This greater variability of the post-irradiation cycles is thus largely due to greater variability of the diœstrous interval, and is well illustrated by the following frequency polygons for length of pre- and post-irradiation cycles :—

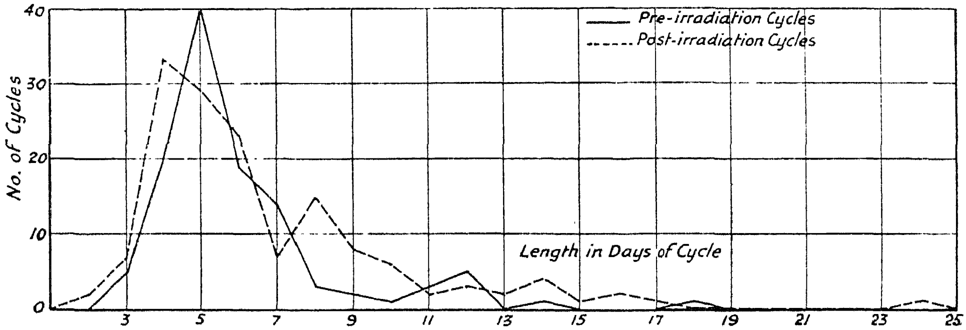

FIG. 1.—Frequency curve for length of pre- and post-irradiation cycles.

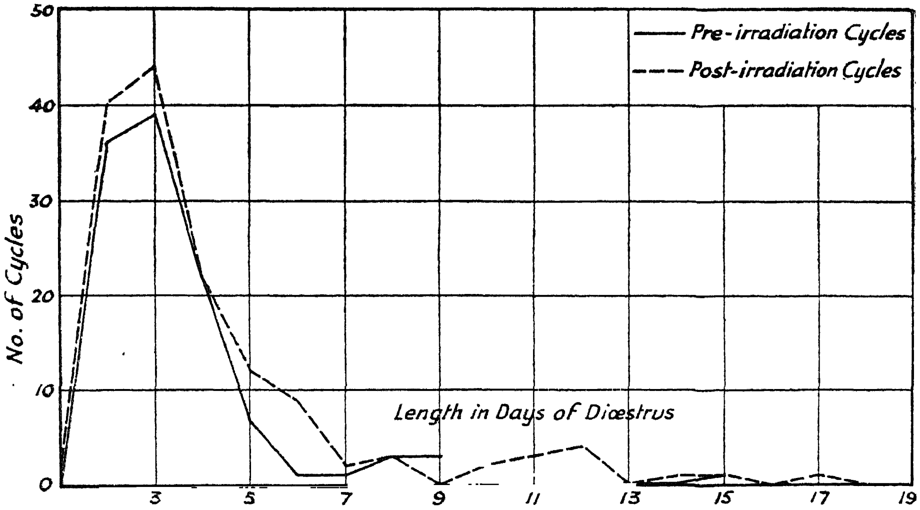

FIG. 2.—Frequency curve for length of pre- and post-irradiation diœstrous intervals.

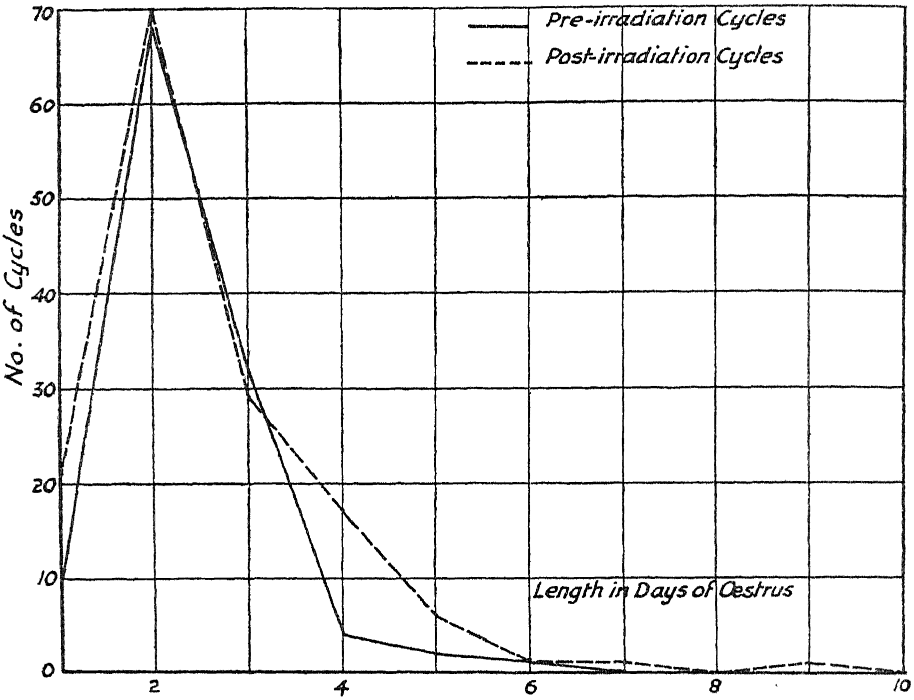

FIG. 3.—Frequency curve for length of pre- and post-irradiation œstrous periods.

The Cycle during the Phase of Follicular Disintegration.—The greater variability of post-irradiation cycles, taken into consideration with the fact that during the first five weeks, at any rate, after irradiation marked changes are taking place in the ovary, suggests that some sort of gradually appearing aberration of the cycle might occur after irradiation. Accordingly, it is necessary to analyse the data from the point of view of the length of each successive cycle after irradiation. In the first place, it might be supposed that the cycle during the time when the follicles are undergoing destruction as organised units would be different from (*a*) the pre-irradiation cycle associated with the normal ovary, and (*b*) the cycle associated with the sterilised ovary after it has reached a stable state. To investigate this point the 146 post-irradiation cycles were divided into two classes, those occurring within five weeks of irradiation and those occurring after.* The results were as follows :—

---

* As an arbitrary method the incidence of the middle day of the cycle was used for this classification. Cycles with an even number of days were classed by the incidence of the first of the two centre days.

Table XVII.—Comparison of Length of Cycle during and after the Period of Follicular Disintegration.

Length of cycle.	Time after irradiation. Number of cycles.		Total.
	0–5 weeks.	6–10 weeks.	
2	1	1	2
3	1	6	7
4	23	10	33
5	20	9	29
6	17	6	23
7	5	2	7
8	9	6	15
9	2	6	8
10	4	2	6
11	1	1	2
12	2	1	3
13	1	1	2
14	2	2	4
15	—	1	1
16	1	1	2
17	1	—	1
24	—	1	1
Total	90	56	146
Mean	6·37 ± 0·204	7·07 ± 0·363	6·64 ± 0·234
σ	2·87	4·03	4·19

Relative to this table the following remarks may be made :—

(*a*) The difference in the mean length of cycle during the first five weeks after irradiation and during the following five weeks is 0·70 days. The p.e. of this difference is $\sqrt{0\cdot204^2 + 0\cdot362^2} = 0\cdot416$. The difference is therefore only 1·68 of its error and is not significant. The numbers of cycles involved are, however, comparatively small.

(*b*) The variability of the cycle, as measured by σ, is greater during the later period.

(*c*) These results, therefore, do not support the possibility that there is any fundamental difference in the length of cycle during the period of follicular degeneration as compared with the later period when the sterilised ovary has assumed a relatively stable state.

Is there a Hiatus in the Cycle at any Time after Irradiation?—Since it is not possible to detect any difference in the duration of the cycle during, as compared with after, the period of follicular degeneration, it is clearly improbable that any definite hiatus occurs at any point, but to complete the analysis of the results it remains to consider the average length of first, second, etc., cycles after irradiation, and as an alternative method of doing the same thing to consider the cycles centering in successive weeks after irradiation. The results are given in Tables XVIII and XIX.

Table XVIII.

Mid-point of cycle (weeks after irradiation).	Number of cycles.	Average length (days).	Number of days occupied.
1	5	6·00	30
2	21	5·66	119
3	25	7·36	184
4	20	4·85	97
5	19	7·53	143
6	16	6·88	110
7	15	5·74	86
8	11	9·36	103
9	6	6·66	40
10	8	7·12	57
Total	146	6·64	969

Table XIX.

Cycle after irradiation.	Number of cycles.	Average length (days).	Days occupied.
1st	20	7·20	144
2nd	20	6·18	131
3rd	20	5·95	119
4th	19	7·31	139
5th	17	6·36	108
6th	15	7·47	112
7th	11	6·46	71
8th	10	6·30	63
9th	7	5·71	40
10th	3	6·00	18
11th	2	6·00	12
12th	1	—	8
13th	1	—	4
Total	146	6·64	969

By neither method is any regular break in the occurrence of œstrus after irradiation to be detected, and it may therefore be concluded that though the post-irradiation cycle has a rather greater variability than the pre-irradiation one, there is no tendency for irregularity to occur at any particular time after irradiation, and no regular hiatus at any definite time attributable to the obliteration of the Graafian follicles can be detected.

(*f*) *Additional Evidence from partially sterilised Animals.*—The statistical discussion concluded above is limited solely to animals known to have been completely sterilised, the object being to include only animals in which no trace of organised follicles was found at autopsy. There were in the series, however, a number of animals which had escaped complete sterilisation and were found on histological examination to have one or more follicles. These animals, though unsterilised in the strict sense of our definition, nevertheless provide valuable evidence that the cyclic structures of the ovary are not essentially responsible for the occurrence of the œstrous cycle. There are seven animals which were kept for upwards of five weeks after irradiation and which were subsequently found to be unsterilised, and in none of these cases were such cyclic structures as were found in the ovary on histological examination sufficient in number or nature to account for the previous œstrous history of the animal on the theory that maturing follicles are essential to œstrus.

The histories of the seven animals are summed up in the following table:—

Table XX.—Histories of Non-Sterile Animals.

No. of animal.	Length of cycles observed before irradiation.		Length of cycles observed after irradiation.		Histology.
	Individual cycles.	Average.	Individual cycles.	Average.	
AX 12	9 days	9	12–3–7–6–5–8–3–6–7–3–4	5·8	One medium-sized and three or four small follicles.
AX 24	7–3–5–5–5–7–7–13–5–7	6·4	5–6–4–3–4–5–6–3–4–7–7–3	4·8	One large degenerating follicle.
AX 31	6–7–7–4–5–4–5–14	6·5	7–13–10–7–13–9	9·8	One or two small, and one large follicle. One or two good corpora lutea.
AX 34	5–5–6–6–2–3–7–6–5	5·0	5–7–5–5 and then practically all cornified	—	Three or four small follicles. Old corpora lutea only.
AX 39	6–5–11–11	8·2	6–4–5–5–5–8–25	8·3	A few follicles and luteal tissue of uncertain age.
AX 48	6–5–6–5	5·5	4–7–5–5–4–7	5·3	One mature and two medium-sized follicles.
AX 50	4–4–5–7	5·0	9–7–5–5–6–6–2–5–6	5·7	Number of follicles, luteal tissue probably atretic.

Only two of these animals showed definite abnormality after irradiation. AX 34 had four normal cycles after exposure and then remained in a state of persistent cornification for 34 days. Since the ovaries contained only a few small follicles and only old corpora lutea, this abnormality is in the opposite direction to what would be expected if œstrous symptoms were dependent on the presence of mature follicles. AX 39 had one abnormally long cycle at the end of the post-irradiation period of observation. No indication of recent ovulation was found in the ovaries, however, and it must be supposed that this long cycle was terminated by an œstrus unaccompanied by ovulation. In general, therefore, it may be said that the few cyclic structures found in the ovaries of these partially sterilised animals offer no adequate basis for the œstrous histories recorded, and that even in these cases of only partially sterilised animals the cycle must have been more or less independent of the cyclic structures of the ovary.

V. *The Duration of Fertility after the Application of a Sterility Dose.*

Since irradiation is followed by the rapid growth of oocytes and possibly by ovulation, it seemed not impossible that pregnancy might result from copulation soon after irradiation, even in animals subsequently becoming quite sterile, and to investigate this possibility a number of animals were mated immediately after irradiation. Of these, eight copulated within one week, while a ninth copulated 20 days after irradiation. Only one of these nine animals was kept for more than 30 days after irradiation, and the ovaries of this one were found on autopsy to be entirely devoid of oocytes and organised follicles. Since the dosage employed totally sterilised 75 per cent. of the animals kept sufficiently long after irradiation to permit of the full effects being shown, it is reasonable to suppose that a similar percentage of these animals would have reacted similarly. Table XXI shows the results of the mating of these animals :—

Table XXI.—Duration of Fertility after the Application of a Sterility Dose.

No. of animal.	Copulation (days after irradiation).	Result.	Date of autopsy (days after irradiation).	Histological findings.
AX 62	1	Normal pregnancy	10	Unsterilised.
AX 61	2	Early pregnancy but re-absorption	23	,,
AX 58	3	No sign of pregnancy	30	,,
AX 25	4	Normal pregnancy	16	,,
AX 59	6	No sign of pregnancy	30	,,
AX 30	6	Normal pregnancy	67	Quite sterilised.
AX 65	7	Litter of one	30	Unsterilised.
AX 66	7	No sign of pregnancy	30	,,
AX 57	20	No sign of pregnancy	31	,,

Of these animals, therefore, four became pregnant following copulation at 2, 4, 6 and 7 days respectively after irradiation. AX 61, copulating at two days after irradiation, became pregnant, but re-absorbed the fœtuses. AX 25 became pregnant following copulation at four days after irradiation, and when dissected 12 days later was found to have nine fœtuses, two in the right cornu and seven in the left. AX 30 copulated six days after irradiation and produced a normal litter of seven (four male and three female) which was suckled normally. It was concluded, therefore, that normal corpora lutea of pregnancy had been produced.

It was thought, however, that since the corpora lutea of lactation are dependent on post-partum ovulation in the mouse, these corpora might not be present, and in an effort to test this an injection of œstrin was given after 10 days suckling. It has been shown elsewhere (Parkes and Bellerby, 12) that in the normal mouse large amounts of œstrin are required to bring the suckling female into œstrus, whereas in the absence of the corpora lutea of lactation a little over one mouse unit only is required. AX 30 was therefore given during suckling three mouse units, an amount insufficient to bring into œstrus the normal female suckling a litter of seven, but sufficient to bring into œstrus the ovariotomised suckling female. AX 30 was not brought into œstrus by the dosage given, so that it must be concluded that the ovaries still retained the œstrus-inhibiting power normally found during lactation, and hence presumably possessed functional corpora lutea.*

Whether there were true corpora lutea of lactation formed after post-partum ovulation or whether the functional life of the corpora lutea of pregnancy had been prolonged by the removal of follicular competition is uncertain, but while the former hardly seems probable, the latter, on account of the normal duration of pregnancy, seems even less so. Soon after the litter was weaned the œstrous cycle of AX 30 began again, and on killing 67 days after irradiation the ovaries were found to be quite devoid of oocytes and follicles. This, therefore, is a perfectly good case of fertile copulation six days after the application of a dose of X-rays sufficient to lead to sterility. None of the young showed abnormal features, and the significance of the observation is difficult to see at the moment, but it seems to offer striking possibilities in the direction of a technique for investigating the function of the corpus luteum.

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* By means of unilateral sterilisation, subsequent removal of the unsterilised ovary, and injection of œstrin, it has been shown definitely what had already been surmised, that the œstrus-inhibiting power of the mouse ovary during suckling depends on the presence of the corpora lutea of lactation.

VI. *Discussion.*

In the previous two papers of this series it has been shown that all the histological and physiological symptoms of œstrus, except ovulation, occur in the complete absence of Graafian follicles and corpora lutea, and in this present paper it has also been demonstrated that the periodicity of œstrus after sterilisation is indistinguishable from the normal. The evidence that the œstrous cycle remains intact after obliteration of the cyclic structures of the ovaries is thus complete. It is necessary, however, to discuss a number of points arising out of the present paper.

Difference in Histological Effects of Irradiation of the Young Animal and of the Adult.—It has previously been shown (2, 3) that irradiation of the young animal results in the entire destruction of the Graafian follicle; ovum, epithelium and theca interna (where it is differentiated) all participating in the general disintegration. In these animals sterilised when young, therefore, it was possible to say that the phenomena of œstrus which were observed occurred in many cases quite independently of tissue of follicular origin. Even if the corpora lutea atretica, and the "anovular follicles" of the second post-irradiation proliferation found in certain cases, be considered as follicular tissue, the statement is still valid, because these were shown to be uncorrelated with the occurrence of œstrus.

The ovary of the animal sterilised when adult, however, differs, as mentioned above, from the ovary of the animal sterilised when young in having a considerable amount of tissue of obviously follicular derivation.

This difference is, however, not of great importance, because it has already been shown that tissue of follicular origin is not essential for the production of œstrus, and in any case the real problem connected with the œstrous cycle of the sterilised animal is not as to what tissue produces "œstrin," but as to how the periodicity is achieved. From this point of view the ovary of the animal sterilised when adult agrees with that of the animal sterilised when young. Neither the tissue of follicular derivation nor any of the tissue of the ovary shows any evidence of cyclic change, so that the real dilemma of how the periodicity is regulated still remains. So far, however, no reason has been found to modify the views expressed in Part II, where it was suggested that the sterilised ovary, and presumably the normal ovary, is regularly producing œstrin, and that the threshold value required to make the stimulus operative is periodically attained.

Relation of the Results to the Œstrous Mechanism in other Mammals.—Whatever tissue produces œstrin in the sterilised mouse ovary and however the periodicity is arranged, one serious difficulty is found. The fact that the normal length of œstrous cycle is found in the mouse after sterilisation, that is to say, in the complete absence of functional corpora lutea, must be considered in relation to the well-authenticated experiments of a number of workers, in which the removal of the corpora lutea of ovulation expedited the appearance of the next œstrous period. Such experiments have been reported by Hammond (4) on the cow, and by Loeb (5) and Papanicolaou and Stockard (8) on the guinea-pig.

How are those experiments on corpora lutea removal to be reconciled with the fact that obliteration of the corpora lutea in the mouse does not alter the periodicity of œstrus? The answer seems to lie in the difference between the cycle in the mouse and in an animal such as the guinea-pig. In the diœstrous cycle of the unmated mouse one œstrus follows another very rapidly, and the cycle consists, in Loeb's (6) terminology, mainly of the follicular phase. In an animal such as the guinea-pig, however, the cycle is much longer, and an appreciable part of the cycle consists, again in Loeb's terminology, of the luteal phase, which is dominated by the corpus luteum. This luteal phase may be regarded as analogous to the definite pseudo-pregnancy phenomena, which are so marked in the rabbit (after sterile copulation) and in the dog, and which culminate, on the pseudo-pregnancy theory, in menstruation in the human subject. It would appear, therefore, that the cycle of the mouse (and presumably also of the rat), since it has no luteal phase to obliterate, remains unaffected by the elimination of the corpora lutea. After sterile copulation, however, in both the mouse (9) and the rat (7), the next œstrous period is postponed, and under these circumstances there does seem to be a luteal phase in the cycle, governed by extended activity of the corpus luteum.

It would clearly be of interest, therefore, to study the effect of X-ray sterilisation on the length of the cycle of the mouse or rat after sterile copulation. Sufficient data are not yet available on this point, but experiments designed to throw light upon it are now in progress. It is, however, of even greater importance that the effect should be determined of X-irradiation and consequent obliteration of follicles and corpora lutea in an animal with a luteal phase in the cycle, such as the guinea-pig. It would seem that in both the guinea-pig and in the cow the cycle should be materially shortened by the elimination of the corpora lutea, and corroboration of a considerable part of the theory of the œstrous cycle could be secured by the experimental confirmation of this hypothesis. Sterilisation experiments on the guinea-pig are now in progress.

In this connection cases of therapeutic sterilisation of the human female are of interest. A large number of cases are on record where X-ray or radium sterilisation has been resorted to for various reasons, but the fact that a large proportion are cases approaching the menopause much reduces the number of cases of value. Nevertheless, there is ample evidence to show that complete destruction of the follicular system of the ovaries of women results in amenorrhœa (13, 14). Dr. Wilfred Shaw, of the Gynæcological Department of St. Bartholomew's Hospital, has very kindly collected for me the histories of a number of cases of women sterilised some time before the menopause was due. Without exception these histories show total absence of menstruation after treatment.

Since the occurrence of œstrous symptoms is not affected by destruction of the follicles, this effect of sterilisation of the human female adds weight to the view that menstruation is in no way physiologically analogous to pro-œstrus or œstrus, and is therefore probably of the nature of pseudo-pregnant degeneration. At the same time, certain authorities, notably Dr. F. H. A. Marshall, definitely dissent from this view. However, whatever may be the precise nature of menstruation, it is clear that a considerable section of the menstrual cycle is dominated by the corpus luteum, and if the conclusions drawn from experiments on the removal of corpora lutea in lower animals may be applied to women, it would seem that the human analogy of œstrus, whatever its nature may be, should occur much more frequently after the elimination of the corpora lutea by X-ray destruction of the follicles. The confusion as to the precise nature of œstrus in women, however, precludes the possibility of practical investigation of this supposition.

VII. *Summary.*

(1) In the two previous papers it has been shown that complete obliteration of the cyclic structures of the ovary of the young mouse by exposure to X-rays does not inhibit the occurrence of œstrus when the animals subsequently become adult, and in the present paper on sterilisation of the adult it is also shown that the periodicity of œstrus is not significantly affected by the destruction of the follicular system.

(2) In the animals used, 116 pre-treatment cycles were observed. These had a mean length of $5 \cdot 98 \pm 0 \cdot 153$ days. The 146 post-irradiation cycles observed had a mean length of $6 \cdot 64 \pm 0 \cdot 234$ days. The difference in length is $0 \cdot 66 \pm 0 \cdot 296$ days, and is thus barely significant. Pre-treatment diœstrous intervals had a mean length of $3 \cdot 64 \pm 0 \cdot 104$ days, while the post-treatment

intervals averaged 4·12 ± 0·160. Here, again, the difference, 0·48 ± 0·190 days, is scarcely significant.

(3) The variability ($\sigma = 4 \cdot 19$) of the post-irradiation cycle is, however, rather greater than that of the pre-irradiation cycles ($\sigma = 2 \cdot 44$).

(4) That œstrus does occur after the destruction of the follicular system of the ovary is thus fully substantiated, and the light thrown by this fact on the theory of the œstrous cycle is briefly discussed, but no necessity has so far arisen to modify the view on this subject expressed in Part II.

(5) The fact that the periodicity of œstrus is not altered in the mouse by the elimination of the corpora lutea is considered in relation to the experiments of Loeb (5) and Hammond (4) on expediting œstrus in the guinea-pig and cow respectively, by the surgical removal of the corpora lutea, and it is concluded that the apparent discrepancy lies in the absence of any marked luteal phase in the cycle of the mouse, whereas in the guinea-pig this phase is the most prominent.

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Studies on the Relation of Gonadic Structure to Plumage Characterisation in the Domestic Fowl.—II. *The Developmental Capon and Poularde.*

By A. W. Greenwood and F. A. E. Crew, Animal Breeding Research Department, University of Edinburgh.

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[Plate 34.]

Introduction.

Among the many sexually abnormal fowls sent to this Department during the last five years by poultry keepers interested in the science of breeding have been eleven birds which together form a distinct class. Instead of developing into normal cocks or hens with appropriate male or female plumage and head furnishings as they approached maturity, these birds assumed, and retained throughout their lives, the characters and behaviour of the fowl completely gonadectomised before puberty. There was never any reason to assume that the underlying physiological inactivity or actual absence of the gonads was other than the result of some developmental disharmony. No sign or history of disease was ever presented. Our knowledge of the adult characterisation of the fowl experimentally gonadectomised when a few days old, allowed us to recognise that in this group of sexually abnormal birds there were genetic males and females in which the gonadic tissues had been absent or functionally insufficient throughout their lives. In one respect only did these birds (save one) differ from the experimental capon and poularde; their combs were not bloodless and scaly, they were bright and healthy-looking, though very diminutive. After having been kept under observation for two years or more, the birds were killed. Post-mortem examination confirmed our anticipations. In all, there was either complete suppression or else very considerable reduction of gonadic tissue. Such cases as these have an important bearing upon the problem of the relation of gonadic structure and function to plumage characterisation in the fowl.

Previous Observations.

The following cases of gonadic suppression and reduction in birds have been recorded: one duck, *Netta rufina*, with imperfect male characters and no gonads (Poll, 1909); four pheasant hybrids, *Phasianus torquatus* × *P.*

colchicus (?) (Smith and Haig Thomas, 1913); *Syrmatiens recessi* × *Phasianus principalis*, reciprocal crosses, all females had small flaccid oviduct but no gonadic tissue (Phillips, 1916); pigeons and ringdoves, pure bred and hybrid, sixteen with no gonads, fifteen in which one gonad was abnormally absent, seventeen in which various degrees of reduction in the size of the gonads were exhibited (Riddle, 1925).

Gonadic suppression and reduction are commonly associated with hybridity (Smith and Haig Thomas's and Riddle's cases). This is particularly well seen among the Lepidoptera. Harrison (1920) found, for example, that all the female offspring of an *Oporabia dilutata* ♂ × *O. autumnata* ♀ mating lacked gonads. Goldschmidt (1916) commonly encountered gonadic reduction in the offspring of European × Japanese crosses of Lymantria. It is also associated with gynandromorphism, as is seen in the cases of Drosophila (Morgan and Bridges, 1919); of Amorpha (Cockayne, 1916); and of Anergates (Adlez, 1908).

Of these authors Riddle alone makes a serious attempt to define the cause of this absence and reduction of gonadic tissue. He argues that all his cases are amenable to the same explanation, being but grades of one and the same process, that the condition is of developmental origin, and that its explanation can only be found in an appeal to the facts of embryology and developmental physiology. He has shown experimentally that a temporary (12–24 hours) functional disturbance (altered oxygen pressure or altered temperature) during the earlier and critical stages of development can lead to the complete or almost complete suppression of gonadic development, and that definite changes in the form of the gonad as well as in that of other organs may result from such changes in metabolic rate. Taking full advantage of the observations of Firket (1914) and of Swift (1914, 1915), who had shown that the primordial germ cells of the chick arise in a crescent-shaped area anterior to the head process and migrate, between the 20–25 somite stages, to collect in the splanchnic mesoderm and blood vessels in or near the region of the right and left germinal ridges; that two to five times as many of these cells congregate in the left germinal ridge as in the right; and that associated with this difference in number of primordial germ cells is the fact that the left gonad of the embryonic chick is larger than the right, Riddle puts forward the following explanation of gonadic suppression and reduction. The size of the gonad bears a definite relation to the number of primordial germ cells that congregated at the site of the gonad. When none of these cells reach the site, no gonad will develop (save perhaps later from a proliferation of the peritoneal epithelium).

Riddle also makes the following observations :—It is quite possible for an oviduct to attain a considerable degree of development wholly apart from the presence of ovarian tissue. Since many of his cases exhibited behaviour characteristic of the male, he suggests that the expression of maleness does not rest upon the internal secretion of the testis, but that the gonad is merely the amplifier of a characterisation, and not its creator.

Description of Cases.

A. Complete absence of gonadic tissue. 1 case.

B. One testis of small size present :

  1. On the right. 1 case.
  2. On the left. 1 case.

C. One ovary of small size present on the left. 2 cases.

D. Two testes of small size present.

  1. With no other obvious developmental abnormality. 3 cases.
  2. With associated somatic or developmental defects. 3 cases.

A.—*White Leghorn.* (Plate 34, fig. 1.)

Weight, 1,914 gms. ; comb diminutive and dry ; comb volume, 2·0 c.cs. ; wattle size, left, 2·5 × 1·75 cms. ; right, 2·75 × 1·75 cms. ; spurs, left, 2·5 cms., right 2·5 cms. ; adrenals, left, 0·16 gm., right, 0·16 gm. ; thyroid, left, 0·08 gm., right, 0·08 gm. ; pituitary, 0·02 gm. ; bone lengths : femur, 10·0 cms. ; tibia, 14·3 cms. ; humerus, 8·7 cms. ; scapula, 8·5 cms. ; coracoid, 6·5 cms. ; furcula, 7·8 cms. ; keel, 14·5 cms.

No gonadic tissue could be identified.

B1.—*White Leghorn.* (Fig. 2.)

Weight, 2,040 gms. ; comb volume, 1·0 c.c. ; wattle size, left, 2·0 × 1·5 cms., right, 2·5 × 0·15 cms. ; spurs, both 2·75 cms. ; adrenal, left, 0·09 gm. ; right, 0·12 gm. ; thyroid, left, 0·065 gm., right, 0·13 gm. ; pituitary, 0·025 gm. ; bone lengths : femur, 9·5 cms. ; tibia, 14·2 cms. ; humerus, 8·8 cms. ; scapula, 8·5 cms. ; coracoid, 6·7 cms. ; keel, 13·4 cms.

Examination of the body cavity revealed no reproductive tissue on the left side. On the right side, in the normal position of the gonad, there was a testis-like organ, weighing 1·13 gms., divided into two lobes by a slight external constriction. The vas deferens on the right side was present as a thin tube, but on the left side no vas deferens could be identified.

Histology of the Gonad.

Posterior Region.—The tissue was composed of rather large tubules in which activity of the constituent cells of the epithelial lining was manifest. Spermatogonia in active mitosis were very numerous, also early meiotic prophases of primary spermatocytes. A few secondary spermatocytes and spermatids were present and very rarely indeed one or two maturing or mature spermatozoa. The lumen of the tubules was filled with cell débris formed by desquamated germinal cells undergoing degeneration.

Anterior Region.—This portion of the gland consisted of a mass of adipose and of tubular tissue. Varying types of tubules were present and may be classified as follows: (*a*) immature spermatic tubules, many of the germinal cells of which were in active mitosis, but there was no evidence of the onset of the meiotic phase; (*b*) characteristic atrophic tubules, the germinal cells of which have not lost entirely the power of undergoing division, although division figures were extremely rare; and (*c*) tubules of an epididymal nature. These tubules were lined by a regular layer of ciliated columnar epithelial cells. The lumen was filled with a faintly staining fluid in which remains of degenerating cells were found. Surrounding the area of testicular tubules there was a thin sheath of fibrous connective tissue. The epididymal tubules for the most part were embedded in a stroma of fibrous tissue external to the testicular tubule area, although isolated tubules, epididymal in character, were found scattered through the central region. At the periphery an isolated tubule with germinal cells in the meiotic prophase (pachytene stage) was found.

B2.—*White Leghorn.*

Weight, 1,815 gms.; comb volume, 2·8 c.cs.; wattles, each 2·5 × 1·5 cms.; spurs, each 2·5 cms.; weight of adrenal: left, 0·14 gm., right, 0·16 gm.; thyroid: left, 0·10 gm., right, 0·07 gm.; pituitary, 0·012 gm.; bone lengths: femur, 9·9 cms.; tibia, 14·0 cms.; humerus, 8·5 cms.; scapula, 8·3 cms.; coracoid, 6·7 cms.; furcula, 7·5 cms.; keel, 13·6 cms.

Dissection showed complete absence of reproductive tissue on the right side of the body. A small piece of glandular tissue (0·016 gm.) was adherent to the posterior margin of the adrenal on the left side.

Histology of the Gonad.

The nodule was enclosed by a thin sheath of fibrous connective tissue, and was divided up into lobules of varying size by invading strands of fibrous tissue from the tunica. The greater part of the gland was composed of tissue that was identical in histological appearance with that of the testis of the immature cockerel, *i.e.*, small tubules, little evidence of branching, single layer of germinal epithelium cells lining the basement membrane, and a well defined central lumen. Very rarely indications of cell activity were noted among the cells of the germinal epithelium. The tubules were packed closely together, the intertubular spaces were restricted and were found to contain capillaries and a supporting framework of connective tissue. Typical interstitial cells could not be identified.

Smaller aggregates of tubules, differing somewhat in structure from those described above, were present. In these, the cells of the germinal epithelium lining the basement membrane were fewer in number, the nuclei were larger and apparently less rich in chromatin, the cell cytoplasm was more abundant and drawn out into fibrillar processes directed towards and partially obscuring the central lumen. There was no evidence of cell activity. These atrophic tubules appear to be formed partly by increased growth of the germinal epithelial cells, which do not undergo cell division, and partly due to the desquamation and degeneration of many of these cells, as is evidenced by the presence of cell débris in the lumen of many of the tubules.

C1.—*White Leghorn.*

Weight, 1,729 gms.; comb volume, 2·0 c.cs.; wattle size, each 2·5 × 2 cms.; spurs: left, 3·0 cms., right (broken), 2·0 cms.; weight of adrenals: left, 0·12 gm., right, 0·110 gm.; thyroid: left, 0·095 gm., right, 0·135 gm.; pituitary, 0·016 gm.; bone lengths: femur, 8·6 cms.; tibia, 12·4 cms.; humerus, 7·5 cms.; scapula, 7·5 cms.; coracoid, 6·0 cms.; furcula, 1·6 cms.: keel, 12·8 cms.; ovary, 5·37 gms.

On examination of the body cavity a fairly large ovary was found with yolks up to about 0·75 cm. in diameter. A few cystic follicles were present, but many of the follicles were macroscopically apparently normal. On the left side, there was a fairly well-developed oviduct. There was no trace of gonadic tissue on the right side of the body. Attached to the left side of the median mesentery there was an oval brownish body, about 1·5 × 1 cms., which on incising exuded a thick yellow yolk-like substance. This is apparently a yolk extruded into the body cavity, which has become attached and encysted.

Histology of the Gonad.

The gonad presented the typical ovarian structure. There was an outer cortical region of stroma in which the oocytes were embedded and an inner fibrous and highly vascular medullary region. There had been apparently rather considerable hypertrophy of the cortical stroma. No primordial or young developing follicles could be identified. Of the larger follicles many were in varying phases of atresia, others had undergone cystic degeneration, while many appeared normal histologically except for the fact that the ovum could not be traced. The follicular contents have taken up the stain, and this may have tended to disguise the presence of the nucleus and its chromatic constituents, which were normally diffuse at this stage of its development. In these follicles, however, the thecæ and the stratum granulosum are apparently quite normal. Packets of typical luteal tissue were found in the normal position in the thecæ of the follicles.

C2.—*Silver Campine.* (Fig. 4.)

This bird was among the first of its kind to be received, and its importance was not clearly recognised. The specimen was preserved as a museum specimen. Diminutive comb and wattles; spurs: right, 1 cm. long; left, 1·5 cms. long.

Post-mortem examination of the body of the bird showed on the left side an ovary with small pale yolks. The oviduct was thin and infantile. No histological examination of the ovary was made. There was no indication of reproductive tissue on the right side of the body.

D1*a*.—*White Leghorn.* (Fig. 3.)

Weight, 2,693 gms.; comb volume, 2·8 c.cs.; wattle size, each 2·5 × 2 cms.; spurs, each 3·5 cms.; weight of adrenals: left, 0·20 gm., right, 0·19 gm.; thyroid: left, 0·04 gm., right, 0·15 gm.; pituitary (incomplete), 0·005 gm.; bone lengths: femur, 9·8 cms.; tibia, 14 cms.; humerus, 8·3 cms.; scapula, 8·4 cms.; coracoid, 6·8 cms.; furcula, 7·5 cms.; keel, 14 cms.

Post-mortem examination showed a heavy deposit of fat, both subcutaneously and periviscerally. Small regularly shaped testes were present, one on each side, in the normal position of the gonads. The right testis was somewhat longer than the left. Left testis, 0·52 gm.; right, 0·64 gm.

Histology of the Gonads.

The testes were essentially similar in histological detail. There was a typical tunica albuginea surrounding a tubular mass. The tubules were small and activity of the germinal epithelial cells was shown by the presence of various mitotic phases in the spermatogonia. Cells in the early stages of the meiotic prophase were numerous, but no stages past the synizetic contraction of the early prophase of the first division were encountered. There was little evidence of desquamation and degeneration of the germinal cells. The constituents of the intertubular spaces were normal.

D1*b*.—*White Leghorn.*

This specimen was amongst the earliest received. Its examination was not so complete. Post-mortem examination revealed paired testes of regular shape. The right testis was much longer than the left. A number of small excrescences from the surface of the right testis gave it a somewhat roughened appearance. The surface of the left testis was quite smooth. A vas deferens was present on each side. Right testis, 0·75 gm.; left, 0·12 gm.

Histology of the Gonads.

Histologically both testes presented in the main the structure of the immature gonad. The majority of the tubules were small. Spermatogonial mitoses were common and in some of the tubules a few early stages of the meiotic prophase were present; no stages, however, later than the pachynema were met with. The intertubular spaces were restricted, and except for the presence of masses of lymphocytes in many of the spaces the intertubular contents were normal. In both testes there were small areas of enlarged tubules in which spermatogenic activity was more marked. This reached a maximum in the right testis, where certain of these tubules were in full activity and in point of size were comparable to the fully functioning tubules of the normal testis. Fully developed spermatozoa were present and the intermediate meiotic stages were abundant. The small raised areas on the surface of the right testis were caused by the onset of spermatogenesis in some of the peripheral tubules. There was no evidence of the presence of luteal tissue in either testis.

D1*c*.—*White Leghorn.*

The examination of this bird was incomplete because the bird was prepared as a museum specimen. Examination of the abdominal cavity post-mortem showed the presence of two small regularly shaped testes. Left testis = 0·58 gm.; right, 0·56 gm. Vasa deferentia present.

Histology of the Gonads.

The testes were similar in histological appearance to those of the immature cockerel. There was a distinct tunica enclosing a mass of small tubules which showed little evidence of branching. In one region of the right testis there was an aggregation of larger tubules, in which many of the cells of the germinal epithelium were in varying stages of mitosis. The onset of spermatogenesis was noticeable in these tubules and early meiotic prophases were numerous. The intertubular spaces were comparatively large, and the contained cells were as those found in the normal immature gonad, except that no trace of luteal tissue could be detected.

D2.—The birds in this section were also peculiar in that each of them was abnormal in form. Very low set, with markedly inturned toes, their appearance and gait were distinctive.

D2*a*.—*Rhode Island Red*. (Fig. 6.)

Weight, 1,418 gms.; comb volume, 0·6 c.cs. Spurs much curled, 3·5 cms. Examination showed two small pear-shaped gonads, with the base of the pear directed anteriorly, one on each side. The vasa deferentia were thin, but distinct. Left testis, 0·0797 gm.; right, 0·0705 gm.

Histology of the Gonads.

The histological structure of the two testes was identical and consisted of a mass of small tubules enclosed by a typical fibrous tunica albuginea. Many of the cells of the germinal epithelial lining were in active mitosis. Sporadic attempts to complete maturation of the germinal elements were shown by the presence in some of the tubules of a few cells in the earlier stages of the meiotic prophase (lepto- and pachy-nema), but later phases were not met with. Towards the central region of these tubules there were many large degenerating cells with pycnotic nuclei, suspended in a clear fluid. These were apparently derived from the cells undergoing an abortive attempt at maturation. The intertubular tissue was normal except that luteal tissue could not be identified.

D2*b*.—*Buff Orpington Crossbred*. (Fig. 5.)

Spurs: right, 2·5 cms.; left, 2·5 cms. As in the previous case, post-mortem examination revealed an accumulation of adipose tissue both subcutaneous and covering the viscera. There were two small regularly shaped testes. Left testis, 0·172 gm.; right, 0·125 gm.

Histology of the Gonads.

Both testes possessed the structure typical of the immature testis. The tubules were small, unbranched, with a definite basement lining of columnar germinal epithelium surrounding a central lumen. The activity of the cells of the germinal layer was shown by the presence of many mitotic figures. There was no evidence in either gonad of the onset of the meiotic phase. The intertubular spaces with the elements contained therein were as in the normal gonad, with the exception that luteal tissue was not identified.

D2c.—*Rhode Island Red.*

Weight, 1,360 gms.; comb volume, 0·4 c.cs.; wattles, each 1·5 × 1·5 cms. spurs, each 1 cm. long. This bird was examined under anæsthesia. Two small testes were found. A piece of the left testis was removed for histological examination.

Histology of Fragment of the Left Gonad.—The tubules were small and of an immature type. Spermatogonial mitoses were not uncommon, and the onset of spermatogenesis was marked by the occurrence of many cells in the early stages of the meiotic prophase. The fact that these cells were commonly present in the lumen of the tubules, many undergoing degenerative changes, indicated that incompleteness of the maturation process was probably due to the removal of these cells from the germinal region of the tubules. No later meiotic phase was noticed.

Discussion.

From the study of these cases the following observations were made:—

1. The amount of testicular tissue present in different cases ranged from none to 1·16 gms. In the individual in which ovarian tissue was present it was 5·3 gms.

2. When present, the gonadic tissue invariably occupied a normal gonadic site.

3. The small testes were in some cases of irregular shape with an uneven surface.

4. Gametogenesis was imperfect, incomplete either throughout the entire gonad or else throughout its greater part. In all cases, save two, in which testicular tissue was present, some phase or other of spermatogenesis was encountered. Spermatogonia were plentifully present. In one case only, however, were tubules at all comparable with those of a normal testis of a mature male found, and in this case these tubules were restricted to a small area of the testis. In all cases in which spermatogenic activity was revealed, the majority of the germinal cells entering the meiotic phase had failed to complete their differentiation and were found lying, degenerate, in the lumina of the tubules. In cases in which the isolated tubules in which spermatogenesis

proceeded to its conclusion were peripherally situated, small elevated areas were found on the surface of the testis, giving to the gonad an irregular and uneven outline. In the case of the ovarian tissue of the developmental poularde, it would seem that oogenesis remained incomplete, since no fully formed yolks were present. Cessation of oogenesis apparently occurs at or about the early meiotic prophase, as is the case in the spermatogenesis of the developmental capon.

5. In the presence of gonadic tissue of the structure and in the amounts mentioned, the plumage of the birds was as that of the gonadectomised fowl. The comb and wattles of the bird in which such gonadic tissue was present, even in the smallest amount, 0·016 gm., differed from those of the completely agonadic bird in that they were vascular and non-scaly, though similarly small.

6. During the life-time of these individuals it was possible, save in the case of the birds with additional somatic abnormality, to distinguish on inspection males from females, the latter being smaller and slighter in build. Examination of their skeletons, though confirming this impression of a sex dimorphism, could not give significant data, because of the fact that the number of the individuals, particularly of the females, was far too small. The agonadic bird was regarded as a genetic male, for the reason that in size and in bone length it resembled the birds with testis far more than the individuals with ovary.

7. Spurs were present in both sexes and were well grown.

8. In several of the White Leghorn capons, reputedly pure-bred, there were feathers in the middle bar of the wing which were deeply tinged with red-gold pigment in their distal halves. Such pigmented feathers have not been encountered by us in the pure-bred normal Leghorn, though they are common in cross-breds.

Riddle's suggested interpretation of his own cases can accommodate certain of these facts. It is perfectly reasonable in the present state of our knowledge to assume that the migrating primordial germ cells can stimulate the epithelium of the germinal ridge, and of this region alone, to appropriate response towards gonad formation, and that the degree of the stimulus is reflected in the size of the resulting gonad. But failure in gonadic function may be due to other causes than failure on the part of the primordial germ cells to reach the germinal ridge, or failure on the part of the epithelium of this region to respond. It is possible that the gonad is normal in its intimate structure and powers of potential differentiation, and that the failure in gonad differentiation is due to the fact that the gonad, during critical stages of its development, finds itself in an internal environment, the body of the bird, which is physiologically imperfect. It may be that the fault lies with some member of the endocrine chain, or with some genetic or developmental derangement in general metabolism.

The fact that in Riddle's cases as well as in the present series there were individuals which exhibited other developmental errors, in addition to those affecting the sex equipment, is possibly not without significance. It is of interest to note, moreover, that the incompleteness of gametogenesis encountered in these cases also characterised the sterile pheasant hybrids described by Smith and Haig Thomas. In the case of these, there exists the possibility that infecundity is due to chromosome incompatibility, whereas in the case of the developmental capon there is no reason to assume that such is a factor in the causation of infecundity. However, it may well be that thorough investigation of the one may lead to an understanding of the other. It is proposed to use a further series of developmental capons for such an investigation.

It is reasonable to argue that if the fault lies with the gonad and not with the soma, then implantation of such gonadic tissue into a normal body will not be followed by the completion of spermatogenesis in that gonad ; whereas if the fault lies not with the gonad but with the soma, then implantation of normal testis from functional cocks into the developmental capon will give results different from those which are known to follow testis transplantation from one normal bird to another, and transplantation of the imperfect gonad of the developmental capon into a normal male will result in complete spermatogenesis in the implant. Further, if the fault lies with the soma, it may be possible so to manipulate the general metabolism of the developmental capon, by means of endocrine therapy or through administration of mineral salts, as to create an internal environment in which the gonadic tissue could flourish and complete its function.

No explanation of the regionalisation of spermatogenesis within the testis can be attempted until a complete study of spermatogenesis in the fowl has been made. Even though Pézard's law of " all-or-none " may hold in the case of normal fowls, and of this there is some doubt, it is certainly the case that in these abnormal fowls there is no clear line of demarcation between some minimum amount of testicular tissue associated with the full development of head furnishings and a subminimal amount with undeveloped comb. In several of these developmental capons there is far more (by weight) testicular tissue than the amount regarded by Pézard as the effective minimum.

If Pézard's law holds, then it cannot be the amount of testicular tissue that is the important factor in comb growth, but the degree of functional activity of that amount, as estimated possibly in terms of completion of spermatogenesis. There would seem to be a connection between the vascularity or non-vascularity of the head furnishings and the presence or absence of cell division in the gonad. This suggestion finds support in cases of abdominal tumour with destruction of the gonad in the fowl.

It is unprofitable to discuss the question of the presence of spurs in these sexually abnormal birds, for as yet nothing is really known concerning the genetics and physiology of spur development.

Summary.

A series of eleven birds is described which throughout their life retained the characters and behaviour of individuals completely gonadectomised before puberty, except that their combs, though diminutive, were bright and healthy looking. Post-mortem examination revealed either complete absence or reduction in the size of the gonads. In such gonadic tissue as was present spermatogenesis was imperfect.

The causes of this gonadic imperfection are discussed. It has not yet been demonstrated whether the imperfection is inherent in the gonad or in the soma.

The expenses incurred during the course of this study were, in great part, covered by a grant from the Government Grants Committee of the Royal Society, and for this we wish to express our thanks.

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DESCRIPTION OF PLATE 34.

Fig. 1.—White Leghorn. Developmental capon. No reproductive tissue present.

Fig. 2.—White Leghorn. Developmental capon. One small testis present on the right side.

Fig. 3.—White Leghorn. Developmental capon. Two small testes present.

Fig. 4.—Silver Campine. Developmental poularde. Small ovary present.

Fig. 5.—Buff Orpington Crossbred. (Dwarf.) Developmental capon. Two testes present.

Fig. 6.—Rhode Island Red (Dwarf). Developmental capon with two testes present.

(It should be noted that the figures do not indicate the relative sizes of the birds.)

A Graphite Suspension for Intravital Injection of Capillaries.

By Cecil K. Drinker, M.D., and Edward D. Churchill, M.D.

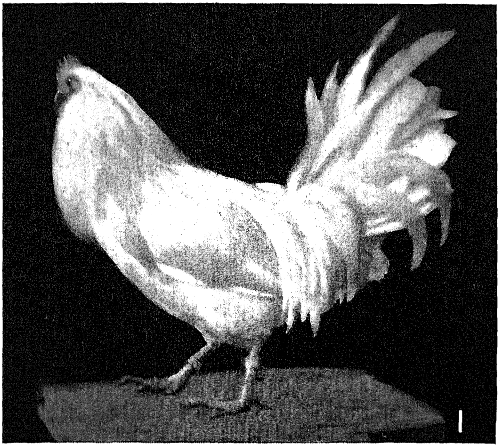
(Communicated by Dr. H. H. Dale, Sec. R.S.—Received April 5, 1927.)

(From the Laboratory of Zoöphysiology, University of Copenhagen.)

[Plate 35.]

In the ordinary use of injection masses the observer expects to fill and obstruct vessels, and the injection is considered successful in the degree to which it seems to fill the maximal number of available paths.

In contrast to this relatively simple procedure is found an increasing number of attempts to inject a living tissue in such a manner as to fill all the vessels transporting blood at the moment when the normal circulation is interrupted. Noteworthy among such applications of injection technique to physiology are the contrasting injections of resting and active muscle made by Krogh (1) in 1919. In these experiments frogs and guinea-pigs received a suspension of India-ink by vein, and this material, mixed with the blood of the animal, was



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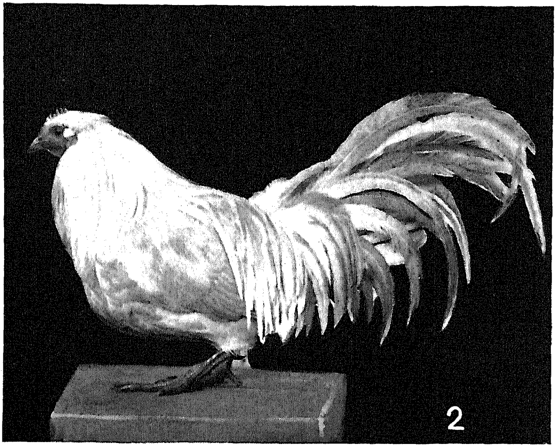

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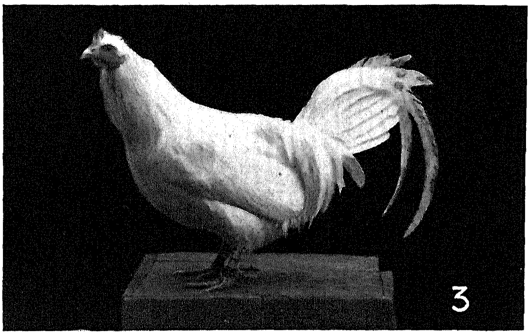

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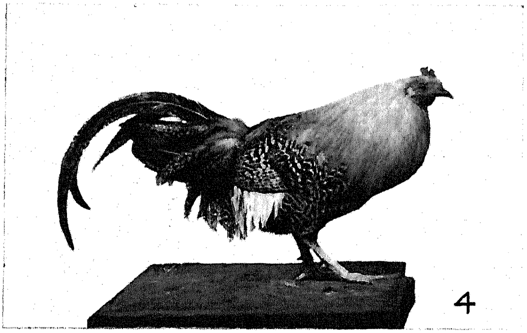

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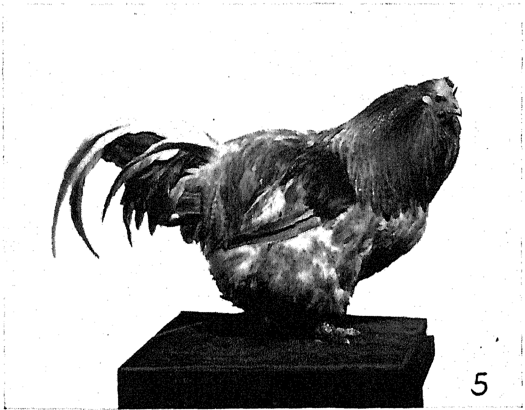

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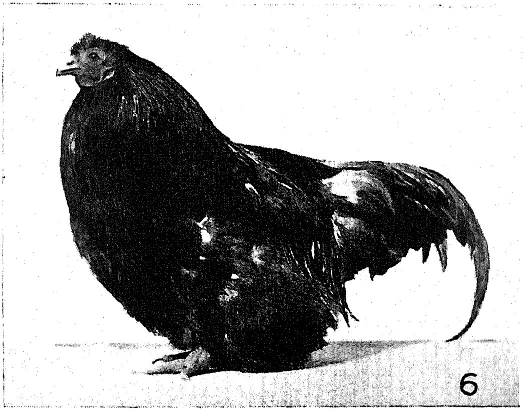

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pumped to the capillaries by the heart. Results obtained in this way constitute single terminal observations, since capillary obstruction begins to take place practically at once.

It became desirable in perfusion experiments upon the frog to make injections which could be removed at will, with return to a clear perfusion fluid carrying substances under examination for their effects upon the capillaries. India-ink is entirely unsuitable for such work, since it has, to a conspicuous degree, a tendency to adhere to vessel walls, an adhesive power which leads to obstructive injections. In order to obtain a true picture of the vascular condition of a tissue at a given moment, it is obvious that a non-obstructive injection will be greatly superior. Indeed, we may consider that the ideal injection mass would be one transported by the capillaries exactly as is the blood of the animal, but so coloured as to permit counts of capillary number, measurement of individual capillaries, etc.

At the suggestion of Prof. Krogh, an ink has been made in which graphite is utilized as the pigment. The results obtained with this ink so far excel those secured with other masses that we have thought it worth while to offer, in advance of further papers dealing with the actual experiments in which it has been employed, a description of this new preparation.

In order to make the ink, one obtains Hydrokollag 300 from E. de Haën, Seelze bei Hannover, Germany. Hydrokollag is a colloidal preparation of graphite containing ammonia and a small amount of cherry gum as a protective colloid. We have obtained it in 100, 500 and 1,000 gm. tins. Number 300 mixes freely with blood plasma, blood serum, and acacia without agglutinating. Other samples of colloidal graphite, prepared and supplied by de Haën, but containing different protective colloids, give gross agglutination or sufficient agglutination to cause their rejection.

The graphite, when received, is a fairly thick syrup with sediment at the bottom. In preparing the ink, this material is first thoroughly stirred and shaken; a portion in grams is then mixed with double the number of cubic centimetres of water, to which has been added enough sodium hydroxide to give a Ph of 8·5. This is sufficient alkali to enable one to drive off the ammonia with an air blast. When ammonia-free, the suspension will show a moderate number of small agglomerations of particles, as it did at the start, and it will have become somewhat concentrated, the degree of concentration depending upon the manner in which the air blast has been employed. In practice it is convenient to dilute 12 gm. of Hydrokollag with 25 c.c. of the sodium hydroxide solution and to allow air to pass through during the night. Next morning, one has from 25 to 30 c.c. of ammonia-free suspension, and to this one adds an equal quantity of 6 per cent. acacia plus 1·3 per cent. sodium chloride, thus securing a final product which contains 3 per cent. acacia and 0·65 per cent. sodium chloride and is suitable for use in frogs. Acacia solutions are ordinarily somewhat acid, and when added in equal amount cause the slightly alkaline suspension to become very nearly neutral. Buffering with phosphates does not affect the suspension. If the ink is to be used in mammals, 12 per cent. acacia and 1·8 per cent. sodium chloride should be employed.

It is next essential to get rid of the aggregates which the preparation contains. The most satisfactory method for doing this is by settling, followed by filtration. The material is placed in high graduates or test-tubes, and is allowed to stand in the ice box for at least twenty-four hours. One may then pipette off the upper portions of the suspension and obtain a material containing comparatively few particles capable of causing embolism. In order to get rid of the few remaining oversize particles, the final product is filtered at least four times through an alundum crucible (R.A. 98, Norton Co., Worcester, Mass., U.S.A.). This crucible is a coarse filter which happens to be exactly right for the final preparation of the ink. It is convenient in that it may be used repeatedly after having been cleaned by firing in the blast flame. No doubt, however, other filters of the required degree of permeability can be found. It is desirable that the filter used should not be too tight. Graphite does not build up easily upon the filter, but even a small degree of plugging makes the final filtration very slow and brings about dilution of the ink.

After four passages through the alundum crucible, the suspension is mixed with an approximately equal quantity of blood serum, blood serum plus acacia solution, or acacia solution alone, the exact degree of dilution desirable being a matter for trial in the region under examination. The final product should show particles of very uniform size and no agglutination.

The size of the individual particles in the mass which we have used, in comparison with human erythrocytes, is shown in fig. 1. The largest particles in such a suspension as this have proved non-obstructive in the frog, but, if desirable, even these may be practically eliminated by lengthening the period of settling. Judicious use of the centrifuge would undoubtedly accomplish the same result, but since our experiments have not required better suspensions than the one illustrated, no attempt has been made to carry the process of sizing to its possible limit.

Plate 35, consisting of four microphotographs of the web of a frog's foot, illustrates the use of the ink. Perfusion of the web illustrated began at 11 a.m., the perfusion fluid being 3 per cent. acacia *plus* 20 per cent. horse serum, with sodium chloride to 0·65 per cent. Picture 1 was taken at 11.25; picture 2 at 11.41, three minutes and forty-five seconds after turning on the graphite ink; picture 3, with lower magnification, at 11.42; and picture 4 at 11.50, nine minutes and thirty seconds after turning off the injection mass and returning to the acacia-serum solution. The melanophores can be seen to have contracted slightly during the period of observation, there having been practically no pituitrin in the solution used. These microphotographs show clearly that the graphite ink has caused no capillary obstruction, and that it has rendered the capillaries beautifully visible.

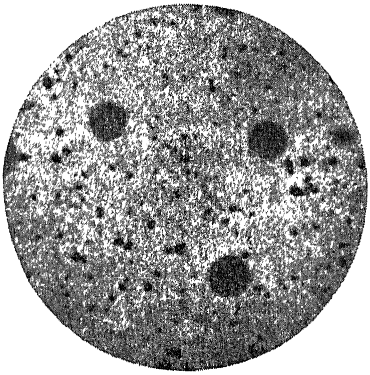


Fig. 1.—Microphotograph of a film of diluted graphite ink suspension mixed with human blood, fixed with methyl alcohol and stained with eosin. Magnification × 286.

Perfusion experiments on the frog's web, lasting just short of three hours, have been performed, during the course of which the injection has been made and removed nine times, without tendency for the graphite to block vessels or to adhere to capillary walls until the last injection, when edema had begun to appear. The longest time that the injection has been kept on in any one experiment is fifteen minutes. This time could, undoubtedly, be exceeded and be followed by complete washing out. When the graphite ink causes obstruction, the result seems rather to express an abnormal change in the capillaries than to indicate any untoward property of adhesion inherent in the graphite. Results of the sort described cannot be obtained by means of any other substance with which we are familiar.

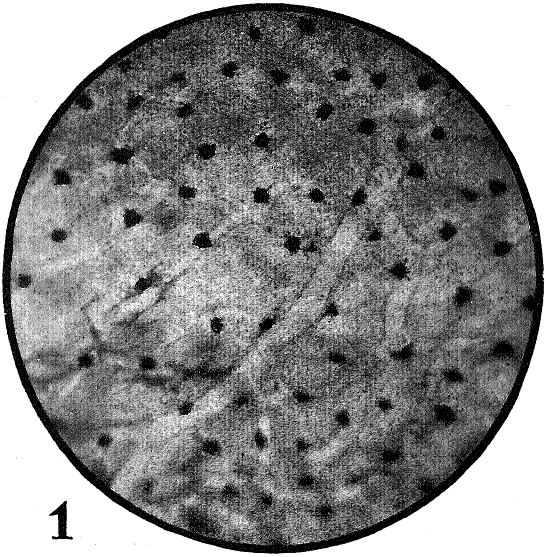
The fact that this preparation of ink contains a small amount of cherry gum has been noted. This gum may be removed by washing with sodium hydroxide solution, Ph 8·5, a washing which will also remove the ammonia. Alundum crucible R.A. 360 worked satisfactorily when used with a pressure of one atmosphere and constant stirring. The procedure is troublesome, and, since cherry gum has proved inert, it may be omitted.

As in the handling of all colloidal preparations, all glassware used in preparing the ink must be scrupulously clean, *i.e.* thoroughly washed in tap-water and rinsed in distilled water before drying. The Hydrokollag, as received, is sterile because of its ammonia content. If handled in a cleanly fashion, mixed with sterile acacia and freshly distilled water, and not allowed to become acid, the final preparation will keep in the ice box unchanged for at least two months.

In addition to the perfusion experiments which have dealt with the foot of the frog, and no other tissues, some observations have been made on the graphite ink injected directly into the circulation. If one arranges a frog so as to observe the capillaries of the tongue and then injects the graphite suspension into the femoral vein, the blood will become black with the particles of graphite, which can be seen running with the corpuscles in the capillaries. No agglutination or blockage is visible, and one apparently has made an ideal inert addition to the blood. But after about ten minutes the graphite particles begin to collect in irregular masses, which appear as if held together by a fine coagulum. These increase in size and soon constitute emboli. The phenomenon is precisely that described by Cohnheim and Litten (2) in 1875 for aniline blue injections observed in the tongue capillaries.

No *in vitro* agglutination of the graphite takes place if the suspension is mixed with heparinized frog's blood and allowed to stand overnight. On the other hand, frogs completely heparinized before the graphite injection show intravascular agglutination, with the same speed and in the same manner as animals whose blood is completely coagulable. The removal of the liver or spleen has no effect in preventing the agglutination. In hepatectomized frogs the number of particles in the circulation remains high for a long time, and one not only has a beautiful view of the agglutination process, but may also follow the gathering of particles by the phagocytes.

The existence of this agglutinative reaction makes it impossible to introduce graphite ink into the circulation with the expectation that matters will remain entirely normal for more than five minutes. In perfusion experiments such as those recounted above, the particles are not recirculated, and the steady washing out seems to avoid the appearance of an effect which would nullify the usefulness of the graphite injections.



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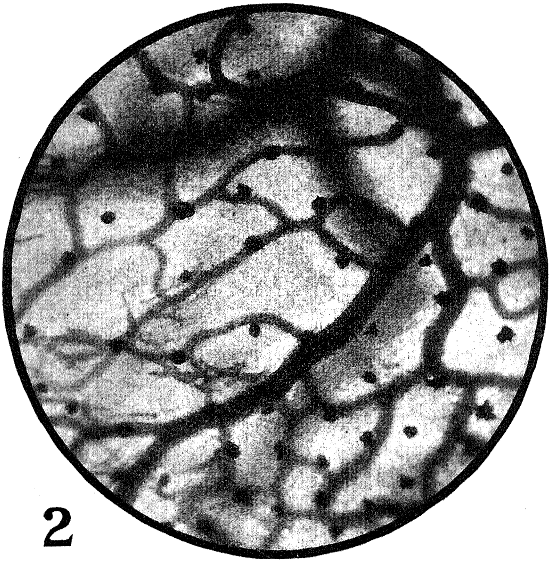

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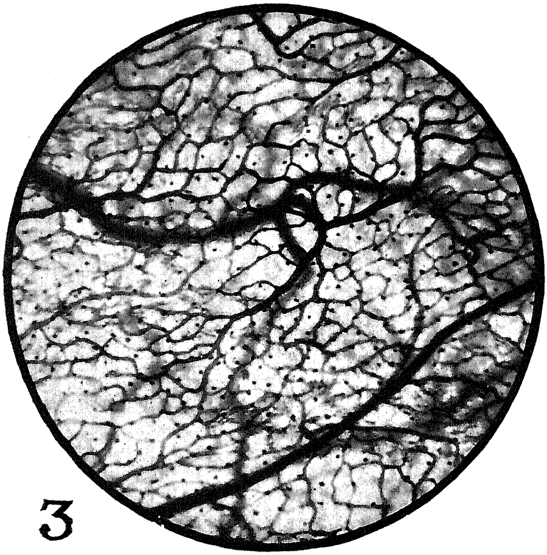

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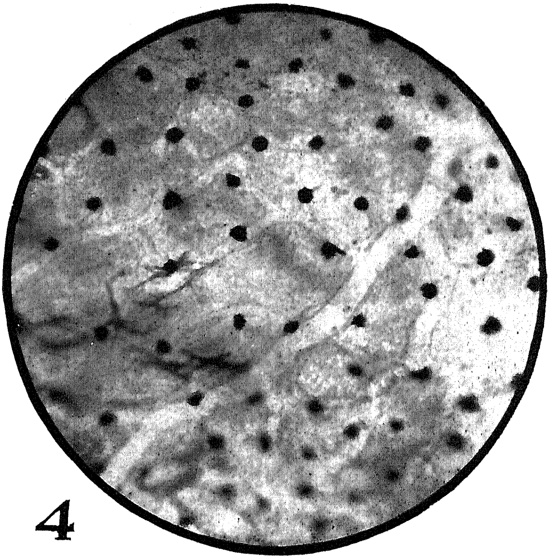

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Summary.

1. A method for preparing a graphite injection fluid is described, and evidence is submitted showing that this fluid possesses qualities essential for physiological injections if employed in perfusion experiments.

2. These qualities consist essentially in the ability of the graphite particles to mix with blood without agglutinating and to pass through the capillaries without sticking to the walls.

3. When injections of the graphite fluid are made in intact animals, intravascular agglutination of the particles begins in about ten minutes and embolism takes place.

It is a pleasure to express our gratitude to Prof. Krogh and Dr. Rehberg.

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(2) Cohnheim, J., and M. Litten, 'Virchow's Arch.,' vol. 65, p. 99 (1875).

DESCRIPTION OF PLATE 35.

Microphotographs of the web of a brown frog. No. 1, taken at 11.25 a.m., after twenty-five minutes of perfusion with an acacia-serum solution, during which a graphite ink injection had been made and washed out; No. 2, taken at 11.41, three minutes and forty-five seconds after turning on the graphite ink; No. 3, taken at 11.42; No. 4, taken at 11.50, nine minutes and thirty seconds after turning off the ink and returning to the clear perfusion fluid. Magnification, Nos. 1, 2, and 4, × 48·4; No. 3, × 16·9.

Summary.

1. A method for preparing a graphite injection fluid is described, and evidence is submitted showing that this fluid possesses qualities essential for physiological injections if employed in perfusion experiments.

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(2) Cohnheim, J., and M. Litten, 'Virchow's Arch.,' vol. 65, p. 99 (1875).

DESCRIPTION OF PLATE 35.

Microphotographs of the web of a brown frog. No. 1, taken at 11.25 a.m., after twenty-five minutes of perfusion with an acacia-serum solution, during which a graphite ink injection had been made and washed out; No. 2, taken at 11.41, three minutes and forty-five seconds after turning on the graphite ink; No. 3, taken at 11.42; No. 4, taken at 11.50, nine minutes and thirty seconds after turning off the ink and returning to the clear perfusion fluid. Magnification, Nos. 1, 2, and 4, × 48·4; No. 3, × 16·9.

On the Nature of "Golgi Bodies" in Fixed Material.

By C. E. WALKER, D.Sc., and MARGARET ALLEN, B.Sc., A.I.C.

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(From the Department of Cytology, University of Liverpool.)

(PLATES 36, 37 AND 38.)

A great deal has been published in recent years upon cytoplasmic structures appearing in fixed material. Among them, those described as Golgi elements, bodies, apparatus and dictyosomes have, perhaps, received most attention. It is unfortunate that these, together with other structures, have been classed as "Cytoplasmic inclusions" (Gatenby, 1917–21, and others). This is a misleading term which obviously does not convey the meaning intended. The structures or bodies referred to are supposed to arise in the cytoplasm, or, being credited with the power of multiplication and development, to be handed on in the cytoplasm from one cell generation to another. The Golgi apparatus is described as being "of very wide distribution among the cells of higher animals, and is known in the Protozoa, everywhere showing the same general characters; and there is reason to believe that the same may be true of plant cells, though considerable doubt concerning this still exists." (Wilson, 1925.) The apparatus or elements may vary from a localised network to scattered granules, curved rods, plates or ring-like bodies.

The Golgi elements are soluble in acetic acid, and hence it has been assumed that they escaped the notice of earlier observers. No acetic acid, a very usual ingredient of fixatives, is used in the treatment of material in which Golgi elements are to be demonstrated. While chondriosomes, in suitably fixed preparations, darken in osmic acid (OsO_4), the Golgi apparatus appears intensely black; hence the two are sometimes supposed to be chemically related. It is claimed that chondriosomes may be differentiated from Golgi bodies by washing the preparation that has been treated with osmic acid in turpentine, when the chondriosomes turn brown, the Golgi bodies remaining intensely black. (Gatenby, 1921.) The chondriosomes, after fixation, are not dependent for their demonstration upon reduction of the reagent used (*e.g.*, OsO_4 or $AgNO_3$), but will stain with certain aniline dyes. Hence it seems probable that there is a definite difference between them and the Golgi elements. [It seems likely that many of the structures produced by the OsO_4 process and labelled chondriosomes are not the same as those demonstrated by other methods. (*April* 12, 1927.)]

There seems to be no evidence justifying the assumption that living protoplasm must have any visible structure. Different kinds of structure may, perhaps, exist in particular kinds of cells, as the foam structure in sea-urchins' eggs (Wilson, 1899); but none of these is general. Moreover, the various kinds of structure described as existing in the ground substance of protoplasm can be manufactured artificially by the action of different fixatives upon colloidal solutions (Hardy, 1899). Comparable differences in the structure of the ground substance of protoplasm follow upon the use of different fixatives. In the course of carrying out the experiments described here many of Hardy's observations have been confirmed.

Bütschli (1894) showed that some of the fibrillar structures seen in fixed preparations of cells were due to pre-existent stresses, and might be reproduced artificially in solutions of albumen. Hardy also produced fibrillar structures in stretched films of colloidal solutions, by fixing them in the same way as is used in fixing cells. The conclusion is that when shear-strains exist in these colloidal solutions at the time of fixation, they appear as definite fibrillar structures in the resultant preparations. A further fact observed by A. Fischer (1894-5-9) is that if two colloids, such as serum albumen and peptone, are in solution together, a fixing agent separates them from each other, as well as from the water with which they are associated. This led Fischer to condemn as artifacts several kinds of granules and bodies appearing in fixed preparations, which have been, and by many are still, regarded as constituents of the living cell. He came to this conclusion because similar structures can be produced by fixatives in solutions containing two colloids (serum albumen and peptone). Granules of a size varying with the strength of the colloidal solution may be produced from a mixture of egg-white, albumose and water by the action of a fixative.

The consideration of these and similar observations naturally suggests the question, how many among the structures we see in fixed preparations are really present in the living cell? That variations in the fixatives used produce very definite variations in the resulting structures in the same colloidal solution, suggest that those structures appearing constantly in similar cells, no matter what fixative be used, are more probably present than those that appear only after the use of a particular fixative. This is probably true to a limited extent, but it must always be remembered that Fischer's discovery, upon which Hardy lays great stress, that in many cases two colloids in solution are separated from each other on fixation, applies not to one fixative only but to many, and also to several different mixtures of colloids.

It would, therefore, not be safe to conclude, because certain structures or bodies appear in cells in a certain physiological phase or state of activity after fixation with several different mixtures in common use, that these structures are necessarily present as such in the living cell. The most that can be said is that there may be substances present which the precipitation or coagulation produced by fixation renders visible. Such a phenomenon may mean no more than that in similar physiological phases the colloids in solution in the protoplasm may be similar in nature and in their relative proportions. The physical characters of the protoplasm of even the same cell may vary widely in different physiological phases and it may safely be assumed that the chemical composition may vary also within limits.

What can be seen in the living cell must be considered differently, but here it is necessary to be very certain that the cell is in a normal condition. Probably the nearest approach to normal conditions, in the case of cells of multicellular animals at any rate, is reached in those which are grown *in vitro*. Tissue cells removed from the body and examined immediately cannot be taken as a safe guide, for it is probable, if not certain, that changes involving the production of structures not existing in the normal cell, set in long before death occurs. Appearances depending upon the use of *intra vitam* stains are also liable to adverse criticism for similar reasons.

By growing tissue cells between a cover-glass and a slide which are separated by some pieces of cover-glass about 12μ thick, we have been able to observe cells under much better optical conditions than can be obtained with cultures on a cover-glass sealed over a hollow ground in the slide. The curved surface of the hollow and the layer of air produce so much distortion that examination of the cells with a high-power objective is very unsatisfactory. We have found that the cytoplasm frequently contains a varying number of granules of different sizes and sometimes vacuoles. In certain cells what are apparently fibrils are very evident. The ground substance of the cytoplasm seems homogeneous and often clear of either granules or fibrils. Some of the granules are probably taken into the cells from the solution in which they are growing, others may have originated in the cells. Some of the smaller show Brownian movement, but no movements such as those described by the Lewises (1915) have been observed.

Fibrils were observed particularly in cells originating from small pieces of the heart of the embryo chick (eight days' incubation). These cells contracted rhythmically, the length being over three times the diameter in the extended condition, less than double when contracted. Fibrils or lines appeared in the direction of the length of the cell, conforming more or less to its outline, the curves being influenced by contraction and extension. [Some short fibrils appeared to show a certain amount of movement independent of the contraction of the cell. (*April* 12, 1927.)] These may have been actual structures or may have been due to shear-strain, which, as mentioned by Hardy (1899), exhibits double refraction in living protoplasm.

The observations, of which a brief account is given above, suggest a very obvious extension of the work of Hardy, Fischer and Bütschli.

Lipins,* particularly lecithin and kephalin, are stated to be constant constituents of cells. Whether they are present as such in the living cell is, for the moment, beside the question. It is sufficient for our purpose to know that they can be extracted from the fresh tissues of animals and plants. Lecithin and kephalin closely resemble each other and are said to be associated in all tissues (Koch and Woods, 1905).

The amount of lecithin varies in different tissues. Young tissues are said to be particularly rich in them (Gilkin, 1907). The testis and pancreas in cattle are stated to contain double the amount found in the thyroid and submaxillary gland, while other tissues vary from each other to an even greater degree, as do also similar tissues in different animals (Cruikshank, 1913, Nerking, 1908, and many others). The amount may vary in different physiological conditions of the same cell (Corner, 1917), and apparently similar specimens may produce very different quantities of lecithin. Again, the degree of unsaturation of lecithin varies greatly in different tissues (MacLean, 1918 (1)) and varies in the same under different conditions (McCollum, Haplin and Drescher, 1912). With regard to this, MacLean (1918 (4)) says " almost all investigators have simply extracted the mixture of phosphatides present and called the extract ' lecithin.' "

Thus, it is clear that the amount of lipins in different tissues is very different, and may vary considerably in the same tissue under different conditions. The variability in the degree of unsaturation of these lipins in different tissues and in the same tissue under different conditions is a very important point in relation to the present experiments, for osmic acid will blacken any given example in direct proportion to its degree of unsaturation. This property is, indeed, comparable to the iodine value, with oxygen from the osmic acid taking the place of the iodine. Another variable factor in the degree of blackening with osmic acid depends on how much oxidation has taken place in the lipins, as the more oxidised the state of the lipin, the less oxygen it can take up from the osmic acid.

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* The term is used to include only the Phosphatides and Cérebrosides (MacLean, 1918).

It also seems probable that the lipins form fat in the cell (Hammersten, 1904, MacLean and Williams, 1909, Heffter, 1891), and that possibly the fat is only a stage in the formation of more complicated compounds. There is evidence to show that part, at any rate, of the lipins forms an association complex with the proteins in the cells (MacLean, 1918 (2)). Lipins are broken up by acetic acid.

In the present experiments lecithin or kephalin, or both, have been added to various mixtures containing egg-white, peptones, proteose, and gelatine in various combinations and proportions. To some, emulsions of cod-liver oil, olive oil, ground up egg-yolk, or methyl myristate have also been added. In order to avoid burdening the text unduly, the exact composition of each mixture is given in the description of the figure which illustrates the appearances produced. The reason for the addition of lipins and fats to mixtures similar to those used by Fischer and Hardy is obvious. Golgi bodies and the lipins are both soluble in acetic acid, and both the lipins and fats blacken with osmic acid according to the degree of unsaturation.

The following general procedure was adopted. The mixtures were spread in films of varying thickness upon slides, and the fixatives were run over the films. Drops of the mixtures were also allowed to fall into the fixatives, when they solidified sufficiently to permit of their being treated in the same way as pieces of tissue. After fixation the films or drops were immersed in a 2 per cent. solution of osmic acid, in the former case for from two to five days, in the latter for a fortnight. The drops were then dehydrated, imbedded in paraffin, cut in sections and mounted on slides. The fixatives used were Mann's fluid,* strong Flemming's fluid,† and glacial acetic one part and absolute alcohol three parts.

Fixation with Mann's fluid and lengthy treatment with osmic acid is claimed to demonstrate Golgi bodies efficiently (Gatenby and Woodger, 1921); so the treatment to which these mixtures were subjected was, in the case of those in which Mann's fluid was used, such as will produce the appearances known as

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* Equal parts of saturated solution of $HgCl_2$ in normal saline and 1 per cent. solution of OsO_4.

† Chromic acid 1 per cent. 15 parts, osmic acid 2 per cent. 4 parts, and glacial acetic acid 1 part.

Golgi bodies in cells; and in the cases where the other fixatives were used, such as will prevent such appearances.

It will, perhaps, elucidate the effect of the introduction of lipins and emulsions to give illustrations of the effect of a fixative upon mixtures of egg-white and peptone, and of gelatine and peptone. The de Witte's peptone used contained about 60 per cent. proteose. It will be seen (figs. 1 and 2, Plate 36) that the two mixtures when spread as films, fixed and stained, produce very similar structures. In the former the small granules are more numerous and more evenly distributed, a few larger spherical ones being present; in the latter the granules are more massed together and tend to be larger.

When lipins are introduced, the mixture fixed, without acetic acid, and the preparation treated with osmic acid, new types of structure appear. The most striking is irregular masses differentiated from the surrounding ground work, with small black granules, curved rods and irregular-shaped bodies on their surface and within their substance (fig. 3). These masses appear less distinct and apparently less numerous in preparations fixed with Flemming's fluid, and there is no sign of the black granules after treatment with osmic acid (fig. 4).

Another new feature in preparations fixed without acid is the appearance of numerous medium-sized spherical granules which blacken intensely with osmic acid. These tend to congregate in groups, particularly round the smaller of the masses just described (figs. 18 and 19, Plate 38). This grouping seems to be more marked in the films than in the drops which have been cut in sections. There are also structures which appear to be masses of granules, rods and irregular-shaped bodies, with, perhaps, the larger spherical granules caught among them (fig. 5). Variation in the proportions of the different constituents of the mixture does not produce a great deal of difference, except in the case of the lipins, and in the nature of the ground substance. Without the peptone the larger spherical granules were not found. Egg yolk was dried, ground up with silver sand, added to the rest of the mixture, and the sand separated off by centrifuging. The result on fixation and treatment with osmic acid was the production of a further variety of bodies, some of which are shown in figs. 6, 7 and 21. Egg yolk treated in the same way and added to gelatine solution produced very similar structures.

The addition of an emulsion of cod-liver oil produced effects such as are illustrated in figs. 8, 9, 10 and 22. Here we have some bodies which are generally brown, but with black structures within them. This effect is accentuated by washing the preparation in turpentine (figs. 8 and 22).

In methyl myristate the fatty acid is completely saturated and so does not blacken with osmic acid. Preparations made with an emulsion of this, added to one of the usual mixtures, shows that black structures of various shapes and sizes may be either spread over the surface of the globules of myristate or may penetrate within (figs. 11–13, 23–25). The form shown in fig. 25 seems more usual when kephalin nearly free from lecithin is used.

When olive oil is used, the preparations, fixed with Flemming's fluid and subsequently treated with osmic acid, show the globules black in the centre but clear at the periphery (fig. 26). In preparations containing an emulsion of olive oil fixed without acetic acid, treated with osmic acid, and left in turpentine for twelve hours, the globules are clear, but some black structures are found round them and a few within (figs. 14 and 15). It will be noticed that, in many cases, the small black bodies are of irregular shape, rods, U's, etc. (figs. 14 and 15).

Where it has been possible, photographs are given, but our preparations present the same difficulties in the way of photography as do those of cellular structures. The shallowness of the focal depth of the objectives, particularly in the case of the higher powers, renders a photograph useless in many cases. In such cases drawings have been made.

Interpretations and Conclusions.

Any suggestions as to what actually happens when these mixed colloidal solutions and emulsions are fixed must, in our present state of knowledge, be more or less speculative. There does not seem to be any necessity to go beyond what Hardy (1899) has said in his admirable paper with regard to the effect of fixatives upon mixtures not containing lipins and emulsions, but the introduction of these substances produces very striking results, which require some further explanation than that offered by Hardy, Fischer and Bütschli for the microscopic appearances produced in their experiments.

In the first place, it is clear that the special appearances we have described are prevented by the presence of acetic acid in the fixative. We know that acetic acid of less concentration than that used in our fixatives will break up the lipins. Hence the structures we have described as appearing in solutions fixed without acid (and which were absent when acid was used), are probably dependent upon the presence of the lipins, the more so as they are absent when neither lipins nor acid are used. The colloidal solutions in which these structures are produced are homogeneous optically, and certainly before fixation neither in them nor in the emulsions is there anything remotely resembling such structures. It seems probable that when the other colloids separate from each other and from the water on fixation, some at least of the lecithin and kephalin is separated, is spread upon the interfaces, and there fixed. It would be unprofitable to consider at present whether, on fixation, they remain as lecithin and kephalin, it is sufficient to assume that they retain some of their fat-like properties and blacken on exposure to the action of osmic acid.

The great variety of structures is easily accounted for. It was not, for the purpose of these experiments, a practical proposition to attempt to obtain pure lecithin or kephalin. When one or the other is specified it means only that one or the other preponderated, probably to the extent of 90 per cent. Commercial lecithin probably contains other lipins besides these two, and was used in those experiments where it is specified. The amount of blackening produced by osmic acid varies with the degree of unsaturation of the lipins, and this varies greatly in different specimens. Lecithin may form compounds or adsorption complexes with proteins and carbohydrates (MacLean, 1918, 3). Proteins darken under the action of osmic acid.

The smaller granules appear and stain readily with the usual dyes, even when acetic acid is used, so do some larger spherical granules. These are almost certainly of the nature of proteins and albumose, as they can be produced by precipitation from solutions containing these, and the precipitates behave in the same way. The granules in the preparations fixed without acid and which are blackened by the action of osmic acid may very well be the same granules, either coated with unsaturated lipin or with which the lipins have formed compounds or complexes. The larger structures are far more complicated. Those such as are shown in fig. 3 seem to consist of an area differentiated from the ground substance, on and within which are distributed small bodies which the osmic acid has rendered intensely black. It seems possible that here we have collections of lipins in which the saturated and unsaturated portions have separated, partly at any rate. It seems also possible that the lecithin and kephalin may, under some conditions, separate in the same way that sometimes happens to fatty substances in the manufacture of margarine.

The small black bodies which are in the form of granules and straight or curved rods, are probably unsaturated, the rest of the structure saturated lecithin or kephalin. It is, of course, probable that the ground substance of these irregular-shaped bodies may be formed of oxidised as well as of saturated lipins, for this would resist the action of the osmic acid as readily as though it were saturated. Another feature of these mixtures containing lipins is that the granules tend to collect round the larger structures, or to collect in groups (figs. 5, 9, 18 and 19).

There are many larger structures in these preparations which are affected by the osmic acid in quite a different way to that described in the group just dealt with. These are either entirely black or contain much larger black bodies than the granules or small rods (figs. 6, 7, 8, 21 and 22). They vary much in size and shape. It may be that they are collections of unsaturated lipins, or of saturated covered with unsaturated. We were led by some of these appearances to introduce a fine emulsion of cod-liver oil into the mixtures, partly to provide globules of fat, which, as we have seen, may possibly be produced by the lecithin and kephalin in the cell, and partly to provide something that might act mechanically in the way the nucleus acts in the cell. Whether there is a nuclear membrane or not, it seems possible that there may be some resemblance between its surface in relation to the cytoplasm, and that of the surface of a globule of fat in relation to the surrounding colloidal mixtures we used. Cod-liver oil is partly saturated and partly unsaturated, and the result of the action of the osmic acid was to produce more and larger forms of black bodies in the preparations. The fact that the globules were rarely entirely saturated prevented any accurate observation with regard to the distribution of the unsaturated, that is the blackened lipins, as it was impossible to discriminate between the black due to the fat and that due to the lipin. Methyl myristate was then tried. It emulsifies temporarily at room temperature and the fatty acid is saturated.

The blackened lipins were sometimes spread over the surface of the globules, and sometimes appeared to penetrate inside. When on the surface they might take the form of strings or groups of granules (figs. 23 and 24), or of very broad loops and lines anastomosing with each other (fig. 25).

It may be that, in the former case, the lipins have combined with the protein or albumose and have not done so in the latter. Again, a peculiar structure in the ground substance which sometimes resembles some of the figures given by Leathes (1925) (fig. 16), and sometimes takes a canalicular form (fig. 17), is more marked where kephalin is present with very little lecithin; so it may be that when the broad anastomosing structure appears, kephalin preponderates over lecithin.

When mixtures containing an emulsion of unsaturated fat (*e.g.*, olive oil) are fixed with Flemming's fluid which contains acetic acid, much of the fat in the larger globules remains unaffected (fig. 26). The appearance suggests that the acetic acid, during the comparatively short time the fat globules have been subjected to its action, has only broken up the fatty acid to a limited depth round the periphery. When, therefore, the preparation is submitted to the action of osmic acid the centres of the globules are blackened. The fixative will probably have coagulated and precipitated the rest of the constituents of the solution before the acid has had time to act upon the fat, thus forming a structure supporting the form of the globule even when the fat is broken up.

When, on the other hand, the same mixture is fixed with Mann's fluid and treated with osmic acid, both fat and lipin are blackened. If this preparation is then washed in turpentine some of the fat is dissolved out, but the lipins remain and are still black. A prolonged washing will cause almost all the fat to disappear, but has comparatively little effect on the lipins (fig. 14).

It is quite clear that the structures representing almost everything that has been published as illustrating the Golgi apparatus, elements, or bodies, may be produced by treating certain colloidal mixtures and emulsions in the way used to demonstrate them in cells. Not only are they similar in microscopic appearance, but they react in the same way to the same treatment upon and after fixation. Both in drops and films these structures appear, but, as one would expect, there is a greater variety in the films, for here there must be more local shear-strains, the effects of which are perpetuated by fixation. The probability that such shear-strains exist from time to time in cells is, perhaps, favourable to the film preparations.

If suggestions as to what happens in these mixtures and emulsions of known composition be speculative, any suggestions as to what happens on fixing cellular structures must of necessity be more so. Some points emerge clearly however. In neither artificial preparations nor in cells do the Golgi bodies appear if acetic acid is used in the fixative. Both are rendered intensely black by the action of osmic acid. Both assume similar shapes and appearances. Both resist the action of turpentine after fixation and osmication (Gatenby, 1921).

In microscopic preparations of tissues we are dealing with the cells as they were when affected by the fixative at the time of fixation and by subsequent treatment. They were dead or on the way to death when they were fixed, and the chemical composition of cells even from the same tissue must vary greatly under different conditions. We know that chemical processes do not cease even after death. It is, however, probable that at the time of fixation the tissues in which Golgi bodies are demonstrated contain several of the ingredients of our mixtures, including the lipins. There is thus ample variation in the chemical composition of the cells to account for the many different shapes and structures of these bodies.

There is a definite indication that the Golgi bodies are most numerous and striking in tissues from which most lecithin and kephalin can be extracted, and where the degree of unsaturation is highest. For example, it has already been pointed out (p. 471) that the testis is particularly rich. The brain and spinal cord are richer, though this, doubtless, is partly due to the myelin substance. According to Cruickshank (1914) the iodine value of lecithin (probably containing kephalin) from the testis was 117, from the heart 72, from the liver 81 and from the thyroid 69. Variations in the iodine value, and, therefore, also in the action of the osmic acid, will be produced also by the extent to which oxidation has taken place. The fixed gametogenic and nerve cells contain very striking Golgi bodies. The canalicular structure, which is by some definitely connected with Golgi apparatus, and supposed to have nothing to do with it by Duesberg (1914 and 1920), is also reproduced very clearly in some of our preparations and may be due to the separation on fixation of the lipins (fig. 17).

The fact that the Golgi bodies can be produced artificially by the same treatment that is necessary to make them apparent in fixed tissue cells, suggests that their appearance may be due entirely to the action of the reagents used upon a homogeneous cytoplasm, and that no structures of the kind exist in the living cell. The Golgi bodies may be unsaturated lipins and the case against them is strong, for the conditions under which they can be demonstrated are extremely limited.

Some of the granules and other structures seen in the living cell may represent chondriosomes. On the other hand, it may be that many of those structures described as chondriosomes in preparations treated with osmic acid are only formed on fixation, but are not composed so largely of unsaturated lipins or fat as the Golgi bodies.

The differentiation of Golgi bodies from chondriosomes by washing in turpentine (Gatenby and Woodger, 1921) suggests very strongly that the one class of bodies consists largely or entirely of lipins, while the other consists of fats or some complex or compound of lipins which is more soluble than the osmicated lipin itself in turpentine. It may be that some of the bodies have only a very thin coating of lipin, and though this is not so readily affected as the fat by turpentine, even a slight degree of solubility would make the body appear brown on prolonged washing. In any case the fact that both lipins, fats and compounds or complexes of lipins and other constituents of the cytoplasm are probably present when the cell is fixed, readily accounts for some of the bodies remaining black and others turning brown, when the results of our experiments in this direction are borne in mind.

The fact that the lipins apparently penetrate globules of fat on some occasions, remaining on the surface on others, will account for many of the appearances presented by Golgi bodies in fixed preparations which have been washed in turpentine.

[These observations show that structures resembling the Golgi apparatus are produced in mixtures containing lipins by the treatment used to demonstrate the Golgi apparatus in cells. The cells are known to contain lipins. If, therefore, the Golgi apparatus is present as such in the living cell, the two structures should both be found in every cell in suitably treated material. (*April* 12th, 1927.)]

There are many structures that were described long before the vogue for Golgi bodies, which appear, no matter what fixatives be used, too complicated and too exactly alike under all reasonable variations of treatment for it to be probable that they are entirely artifacts. The vesicles which, in the spermatid, have been traced as developing through an elaborate process into the caps or acrosomes of the spermatozoa, form an example (Benda, 1896, Baumgartner, 1902, Moore and Walker, 1906, Walker 1925, and others). The various stages appear, no matter what the fixative or method of staining, no matter what the structure produced in the ground substance. [These vesicles, which appear not only in the spermatid but also in the prophases of the meiotic divisions, have been described as arising from the Golgi apparatus (Gatenby and Woodger, 1921), and it is possible that claims to have observed the Golgi apparatus in living cells may be based upon the observation of these structures; though the reason for calling them Golgi apparatus is not clear, as their existence, function and destiny has been known for thirty years. (*April* 12, 1927.)]

At first sight it might appear that all this is simply destructive criticism. Apart from the fact that, if these observations are correct, no addition to knowledge can be entirely destructive, it seems possible that these investigations may facilitate a further advance in our knowledge of various processes taking place within the cell. Besides this, very great importance has been attached to the Golgi bodies by many writers. A "Pathology of the Golgi Apparatus" has been published (Cowdray, 1924) by which the writer evidently means the variations of the Golgi apparatus of cells in different pathological conditions. These include many forms of cancer, tubercular conditions, the after-effects of operations, particularly upon nerves, and changes said to follow the administration of poisons.

Though it would seem that much of the work involved in making these very numerous observations upon Golgi bodies in fixed tissues has been in vain, it may happen that some modifications of an elaborate technique devised to demonstrate something that probably does not exist as such in the living cell, will be of value as a means of advancing our knowledge of chemical changes taking place in cells under different physiological and pathological conditions.

It may be that herein lies a method of distinguishing fat from lipins within the cell, and, after a further development of technique, of estimating the amount of lipin in the cell itself. The amount of lipins extracted from tissues cannot, in some cases, have any relation to the amount in the individual cells; for instance, in the white matter of the central nervous system a large proportion of the lipins is in the myelin substance. Again, there are indications that the changes in the "Golgi apparatus" described as following upon phosphorus poisoning and the section of nerves, are due to changes in the fat and lipin content of the cells. Some of the illustrations suggest that the lipins or fat have become saturated or oxidised and increased in amount. (Cowdray, 1924.)

We have to thank Mr. Clifford Dobell for helpful advice, and Prof. Hilditch not only for advice but also for supplying us with the methyl myristate used in these experiments. We are indebted to the British Empire Cancer Campaign for a grant which enabled us to carry out the work.

Summary.

1. The Golgi elements and apparatus, and some of the structures described as chondriosomes in preparations treated with osmic acid, are probably artifacts produced by the methods used to demonstrate them.

2. Hardy and others have shown that the ground substance of protoplasm, as seen in fixed preparations, may be reproduced by treating certain colloidal solutions in the same way as the protoplasm.

3. Golgi elements, bodies and apparatus do not appear in microscopic preparations if acetic acid is a component of the fixative used. After fixation without acetic acid, they blacken when subjected to the action of osmic acid.

4. If lecithin and kephalin are added to certain colloidal mixtures, and films or drops of the mixtures are fixed without the use of acetic acid, structures exactly like Golgi elements, bodies and apparatus are produced.

5. These structures do not appear if acetic acid is used in the fixative. Lecithin and kephalin are broken up by acetic acid. Both are found in all cells.

6. These artificial structures behave in the same way as the Golgi apparatus when treated with osmic acid, and when washed with turpentine.

7. When a fine oleaginous emulsion is added to the colloidal mixtures, the

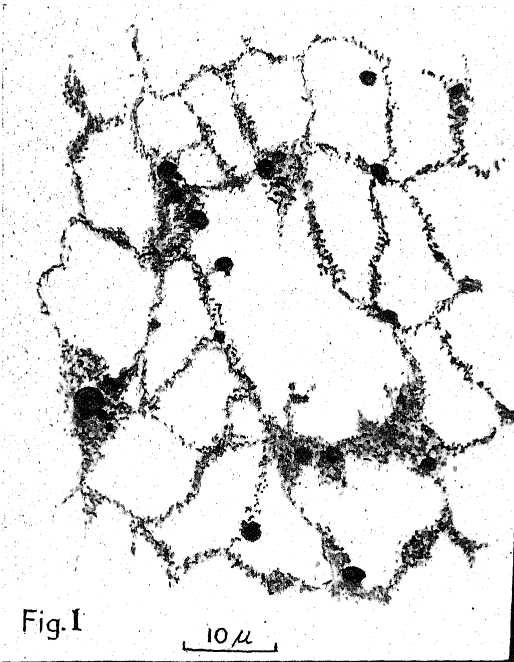


Fig. 1

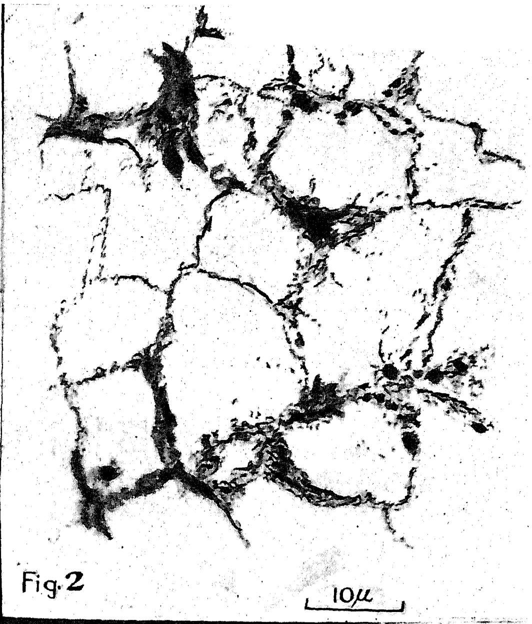


Fig. 2

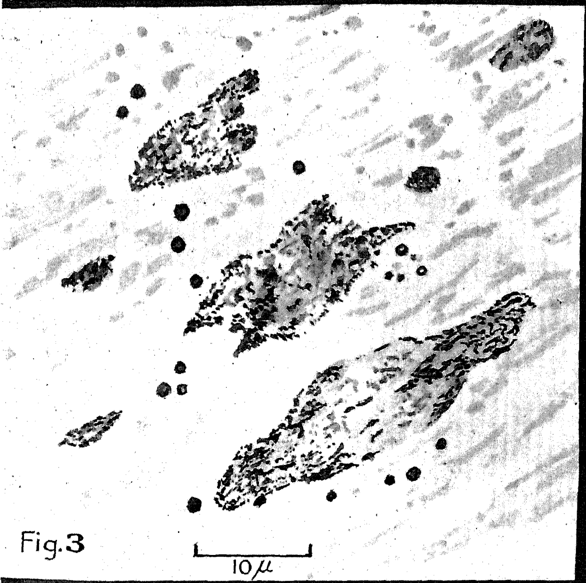


Fig. 3

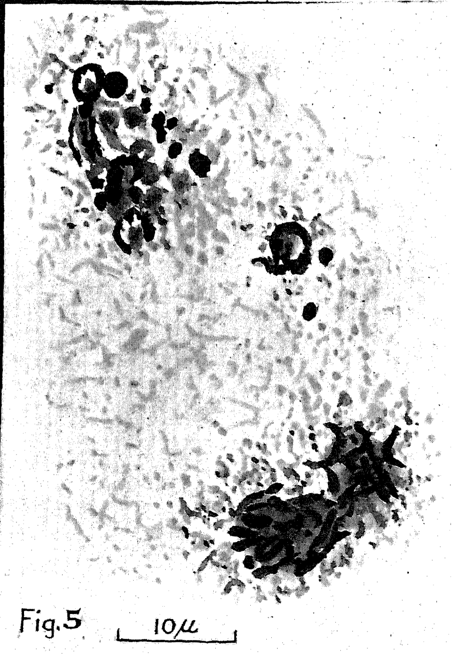


Fig. 5

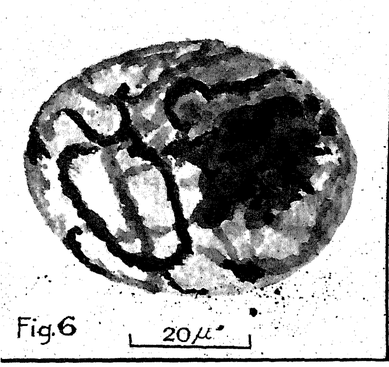
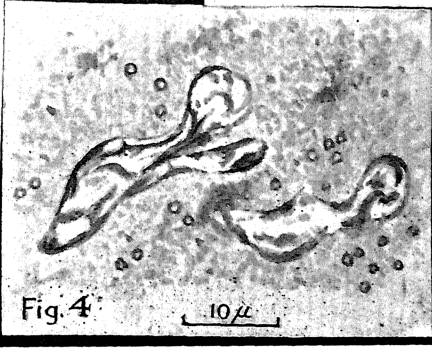


Fig. 6



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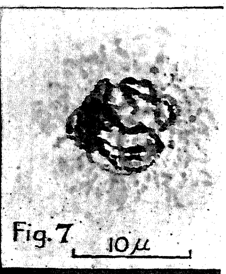

Fig. 7

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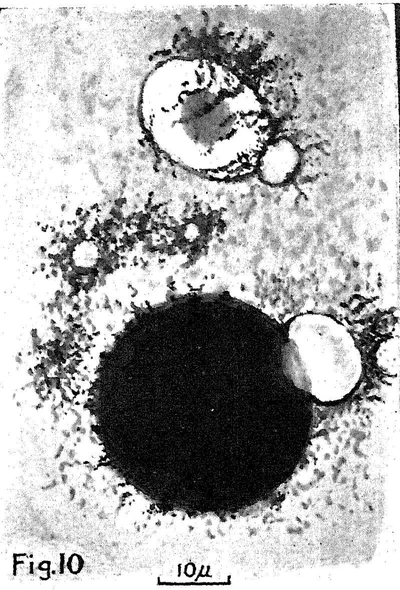
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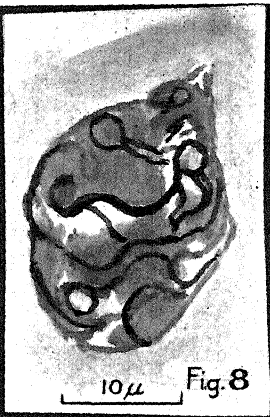
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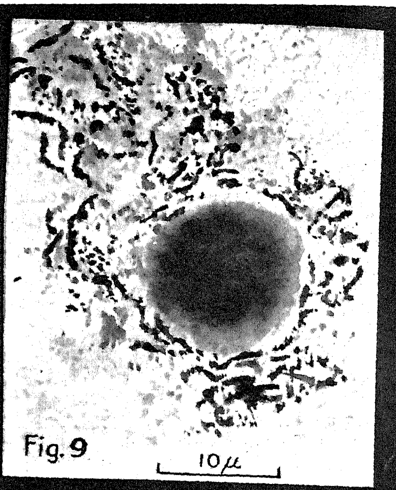
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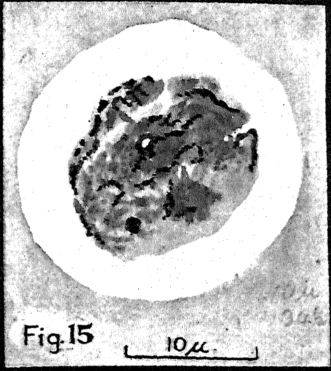
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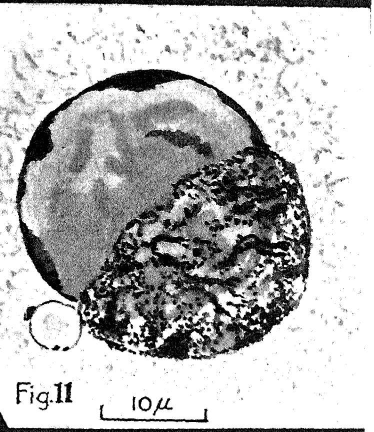


Fig. 11

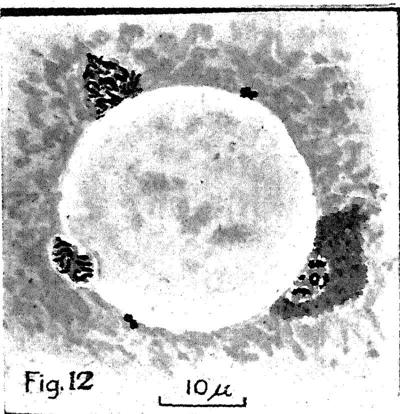
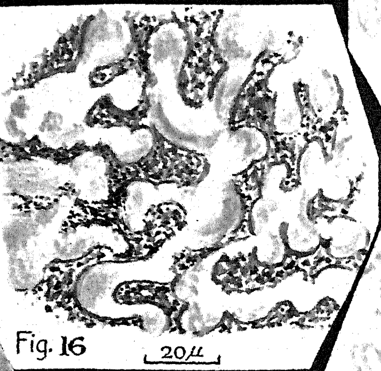


Fig. 12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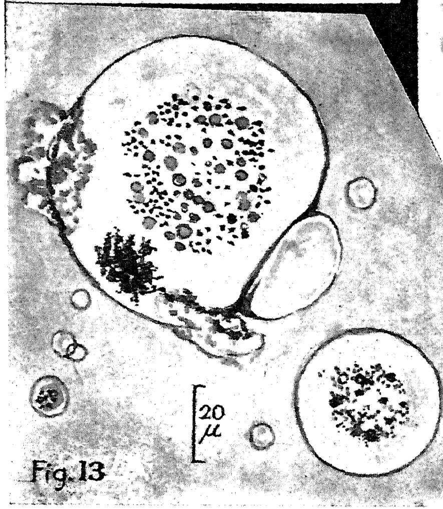
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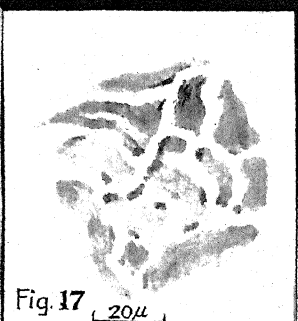
Fig. 17

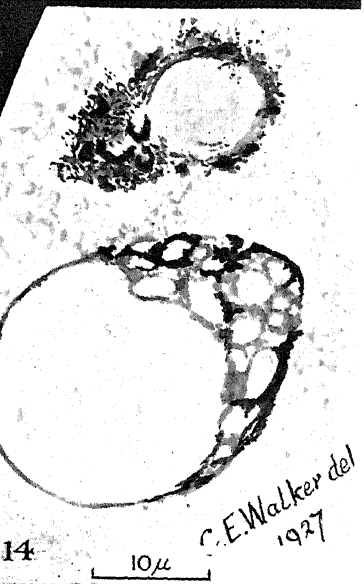
Fig. 14

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structures produced by fixation and osmication appear to behave in a similar manner in relation to the globules of the emulsion as the Golgi elements to the nucleus of the cell.

DESCRIPTION OF PLATES.

N.B.—In the case of egg-white and egg-yolk, the amounts are probably not quite exact.

PLATE 36.

FIG. 1.—Film. Egg-white, 1 per cent.; de Witte's peptone, 0·5 per cent. in water. Fixed, Flemming. Stained, methylene blue and orange G.

FIG. 2.—Film. Gelatine, 1 per cent.; peptone, 0·5 per cent. in water. Flemming. Basic fuchsin and orange G.

FIG. 3.—Film. Egg-white 9 per cent.; gelatine 1 per cent., lecithin extracted from sheep's brain with cold ether, precipitated and washed with acetone, 0·6 per cent. in water. Mann. Osmic acid 2 per cent. 4 days. No stain.

FIG. 4.—Section of drop, mixture as in fig. 3. Flemming. OsO_4. 14 days. Basic fuchsin.*

FIG. 5.—Film. Same as fig. 3.

FIG. 6.—Film. Egg-white 9 per cent. Gelatine 1 per cent. Ground egg-yolk 75 per cent. in water. Mann. OsO_4. 5 days. No stain.

FIG. 7.—As fig. 6.

PLATE 37.

FIG. 8.—Film. Egg-white 7·5 per cent. Gelatine 1 per cent. Peptone 0·5 per cent. Lecithin (commercial B.D.H.) 0·2 per cent. Cod-liver oil 1 per cent. in water. Mann. OsO_4 2 per cent. 4 days. Washed in turpentine 6 hours.

FIG. 9.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·5 per cent. Kephalin† 0·25 per cent. Cod-liver oil 1 per cent. in water. Mann. $2OsO_4$ per cent. 4 days. No stain.

FIG. 10.—As fig. 9.

FIG. 11.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·75 per cent. Lecithin‡ 0·3 per cent. Methyl myristate 0·7 in water. Mann. OsO_4 2 per cent. 5 days. Basic fuchsin.

FIG. 12.—As fig. 11, but not stained.

FIG. 13.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·5 per cent. Lecithin (commercial) 0·2 per cent. Methyl myristate 0·5 per cent. in water. Mann. OsO_4 2 per cent. 4 days. No stain.

FIG. 14.—As fig. 13, but washed in turpentine for 12 hours.

FIG. 15.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·75 per cent. Lecithin (as in fig. 11), 0·3 per cent. Olive oil 1·5 per cent. 2 per cent. in water. Mann. OsO_4 2 per cent. 4 days. Washed in turpentine 10 hours.

---

* The figures could be seen quite distinctly under the microscope, but were fainter than shown in the drawing.

† Kephalin was prepared by drying egg yolk at a low temperature, extracting with ether, and precipitating with acetone. The lecithin was extracted from the precipitate by shaking with warm alcohol, leaving the kephalin which was washed, etc.

‡ The lecithin extracted in process of preparing the kephalin already referred to.

FIG. 16.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·5 per cent. Kephalin 0·2 per cent. Methyl myristate 0·5 per cent. (circ.), in water. Mann. OsO_4, 2 per cent. 4 days. Basic fuchsin and orange G.

FIG. 17.—As fig. 16. In both this and fig. 16 the outlines have been accentuated in the drawings.

PLATE 38.

FIGS. 18 and 19.—Films. As figs. 3 and 5.

FIG. 20.—Section of drop, mixture as in fig. 3. Mann. OsO_4 2 per cent. 14 days.

FIG. 21.—Film as fig. 6.

FIG. 22.—Film. Gelatine 1 per cent. Lecithin (commercial) 2 per cent. Codliver oil 1 per cent. in water. Mann. OsO_4 2 per cent. 4 days. Washed with turpentine 6 hours.

FIGS. 23 and 24.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·5 per cent. Lecithin (commercial) 0·2 per cent. Methyl myristate 0·5 per cent. in water. Mann. OsO_4 2 per cent. 4 days. No stain.

FIG. 25.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·5 per cent. Methyl myristate 0·5 per cent. (circ.). Kephalin 0·2 per cent. in water. Mann. OsO_4 2 per cent. 4 days. No stain. (N.B.—It was impossible to have all the surface of the globule in focus together, as the 3 mm. apochromat. Zeiss had a very shallow focal depth. By focussing it could be seen that the anastomosing network was continuous.)

FIG. 26.—Film. Egg-white 7·25 per cent. Gelatine 1 per cent. Peptone 0·75 per cent. Lecithin (as in fig. 11) 0·3 per cent. Olive oil 1·5–2 per cent. in water. Flemming. OsO_4 2 per cent. 3 days. No stain.

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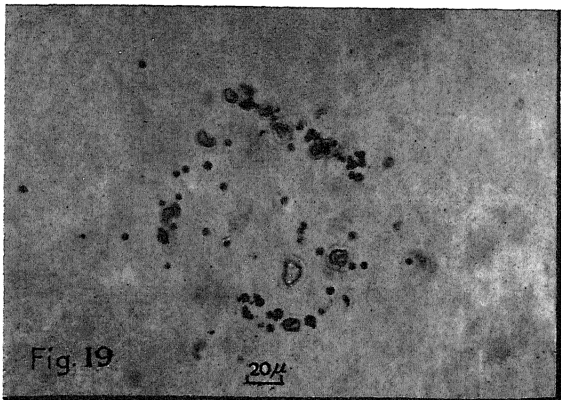
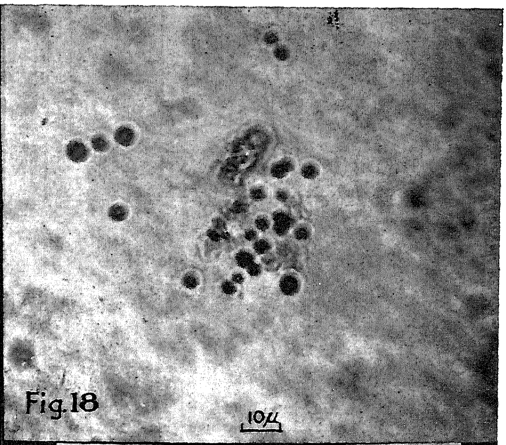


Fig. 19



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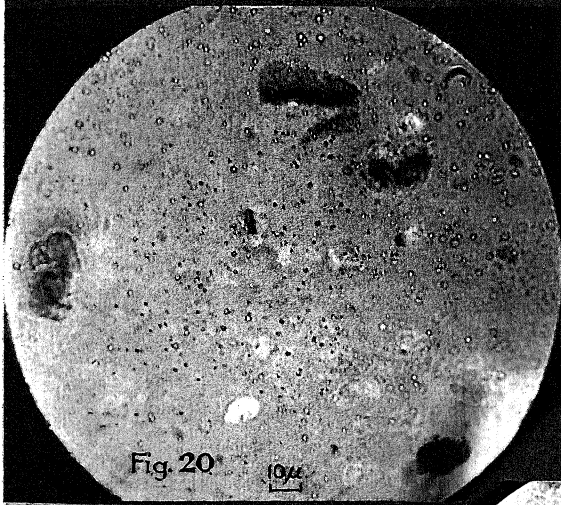
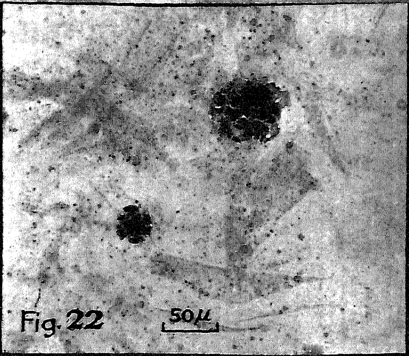


Fig. 20



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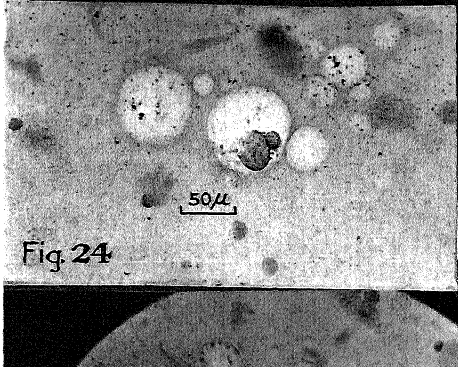

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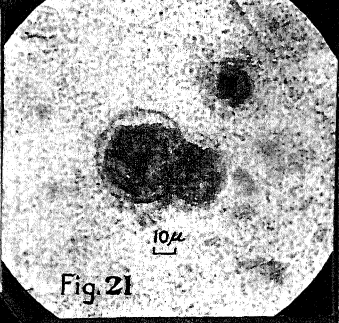

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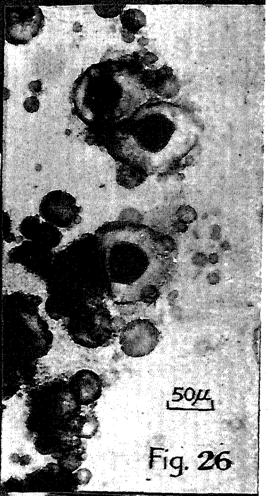

Fig. 26

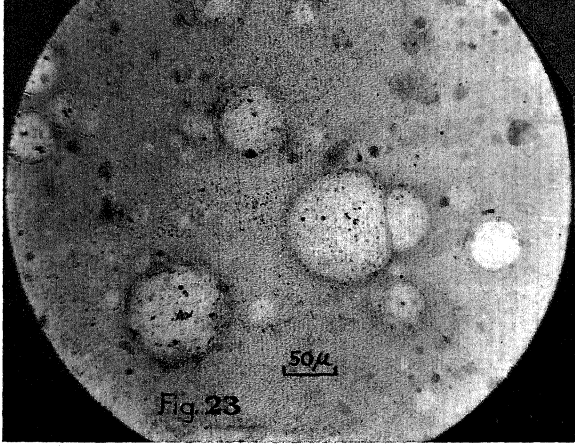

Fig. 23

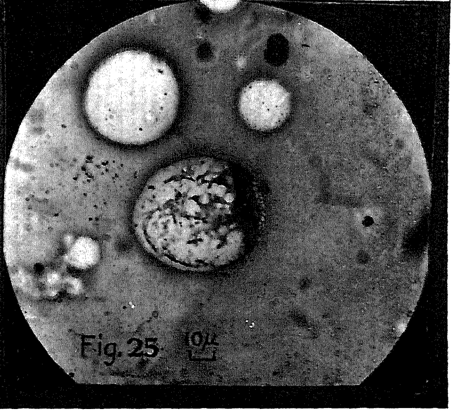

Fig. 25

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The Error of Determination of Toxicity.

By J. W. Trevan, Wellcome Physiological Research Laboratories, Beckenham, Kent.

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I. *The Meaning of the "Minimal Lethal" (or Effective) Dose.*

The determination of toxicity is usually given quantitative expression by the statement of a minimal lethal dose. The common use of this expression in the literature of the subject would logically involve the assumptions that there is a dose, for any given poison, which is only just sufficient to kill all or most of the animals of a given species, and that doses very little smaller would not kill any animals of that species. Any worker, however, accustomed to estimations of toxicity, knows that these assumptions do not represent the truth. How widely different is the real state of affairs, however, is not, I think, sufficiently recognised. The fact that the "minimal lethal dose," whether calculated for unit weight, or for surface area, or on any other basis, varies widely for different species has, perhaps, led to the looseness of its definition for any one species. For the accurate standardisation, by biological methods, of drugs which are not available in chemically pure form, it is essential to establish a more accurate definition of such terms as "minimal lethal dose," "minimal effective dose," etc.

Fig. 1 gives the results of the injection of four poisons into animals. The abscissæ are proportional to the doses injected, the scale obviously differing for the different drugs, and the ordinates give the percentage mortality for each dose injected. The number attached to each observed point represents the number of animals injected for its determination. The curves represent percentage mortalities produced by the subcutaneous injection of tincture of digitalis into frogs, by the intravenous injection of cocaine hydrochloride into

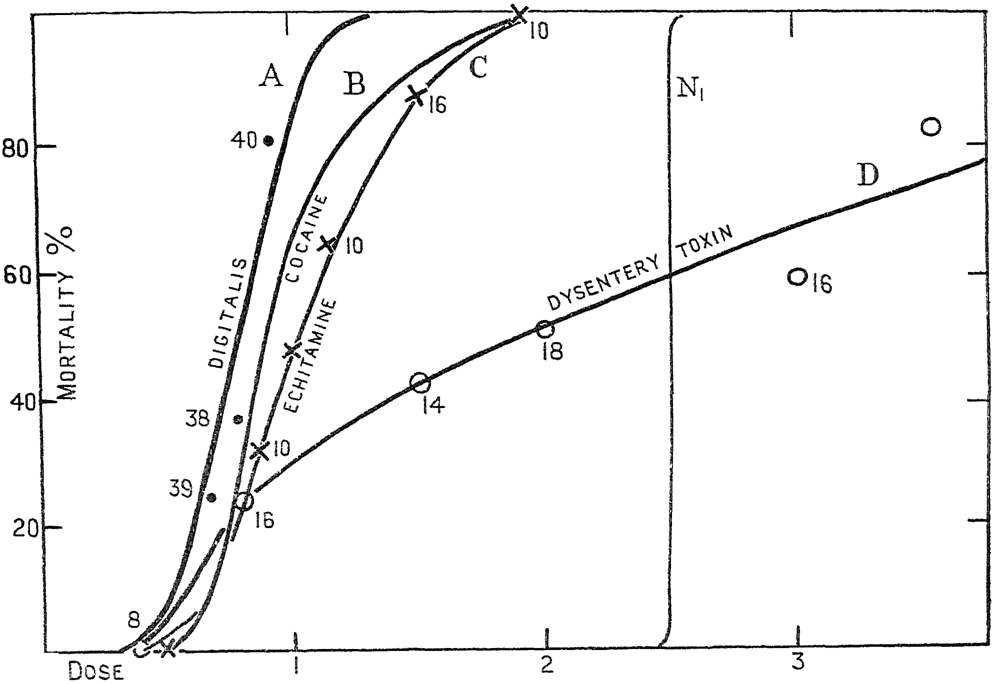

Fig. 1.—Mortality-dose curves (characteristics) of four poisons. A.—Digitalis on frogs. B.—Cocaine on mice. C.—Echitamine on Mice. D.—Dysentery toxin on mice (O'Brien, Sudmersen and Runge). The dose scale has been adjusted for each, so that the steepest parts of the curves are above one another. The numbers attached to the observed points represent the number of animals used to determine that point. N_1 represents the "ideal" characteristic (see text).

mice (see also fig. 2 and Table I), by the intravenous injection of echitamine into mice, and by the injection of dysentery toxin into mice, the data for the last being taken from O'Brien, Sudmersen and Runge (1924). A similar curve is given later (fig. 7) for the percentage of convulsions produced in mice by increasing doses of insulin, the data being obtained by the use of large numbers of animals. Shackell (1925) has published a number of similar curves, relating percentage mortalities to varying doses of different poisons, in a wide range of species. It is suggested that the curve expressing the percentage of mortality, or of some other limiting biological effect, produced by varying doses of a drug on animals of a certain species, shall be called the "characteristic" for that particular drug, effect and species. Thus, the curve relating the percentage of convulsions produced in mice to varying doses of insulin, would be termed the characteristic for the production of convulsions in mice by insulin.

Table I.—Mortalities after different doses of cocaine hydrochloride injected intravenously into mice kept at room temperature. Doses varied in proportion to weight. Variations of dose obtained by increasing or diminishing the volume of the 0·1 per cent. solution of hydrochloride injected. Dose given in terms of dose for 20 grammes of mouse.

Dose for 20 grammes.	Number of mice injected.	Number killed.	Percentage Mortality.
Mgms.			
0·8	20	20	100
0·7	24	20	84
0·7	20	17	
0·6	48	37	78·2
0·6	30	24	
0·5	65	31	55·25
0·5	30	19	
0·4	30	5	15·93
0·4	39	6	
0·3	20	0	0

It is quite clear from these curves that the lack of accurate definition of the term "minimal lethal dose" or "minimal effective dose" may lead to widely different estimates of the toxicities of any of these four poisons. The value indicated by different authors as the "minimal lethal dose" may be either the dose just sufficient to kill only an occasional animal, or that which kills 50 per cent., or that which is just large enough to kill all the animals. The first probably approximates to what is sometimes called the "maximum tolerated dose," and the last is the "certainly lethal dose." These two doses are approximately in the ratio of 1 to 4·8 for digitalis on frogs, 1 to 3·85 for cocaine on mice, 1 to 5·5 for echitamine on mice, and about 1 to 20 for the dysentery toxin on mice. It is clearly necessary to define more closely what is meant by "minimal lethal dose," if the term is used as a measure of toxicity.

The choice of such a measure will be rendered easier by considering the nature of a dose-mortality "characteristic." Shackell has pointed out that these curves are integrated frequency or percentile curves. The sloping curves owe their shape to the fact that different individuals of a given species require different quantities of the poison to kill them. The curves are obtained by injecting groups* of animals with different doses of the poison under investigation, noting the percentage mortality at each dose, and drawing a smooth curve through the points so obtained.

The animals which die in any group, such as those represented by the point labelled 48 in fig. 2, are not only those in that group for which the dose injected is that just sufficient to kill, *i.e.*, those for which it is the individual lethal dose, but also those for which the dose just sufficient to kill is smaller than the dose injected. The smaller the range of variation among the individual lethal doses, the more nearly would the curve approximate to a vertical straight line, such as N_1 in fig. 1.

It is impossible to determine directly for single animals the "individual lethal dose," but approximations to its determination may be obtained in two ways.

(1) Increasing doses, separated by sufficient time intervals, might be administered to the group of animals, and the dose noted at which each animal dies. If the increments of dose were sufficiently small, an individual lethal dose could thus be determined for each animal. Variations, however, probably occur in the individual lethal dose from day to day, which would invalidate the method, and the intervals between the successive doses necessary to prevent the effects of partial accumulation, would render the method impracticably consumptive of time.

(2) A method of continuous slow infusion, such as that used in the Hatcher cat-method for digitalis, gives an approximation to the individual lethal dose.

---

* In the following discussion, to avoid confusion, the words "groups of animals" are used in the statistical sense of "random sample," whilst the word "sample," where used, refers only to a sample of poison.

The dose which finally kills is somethat larger, however, than the true individual lethal dose, if there is a latent period in the action of the drug.

The *distribution* of the individual lethal doses in one of the forms of the usual

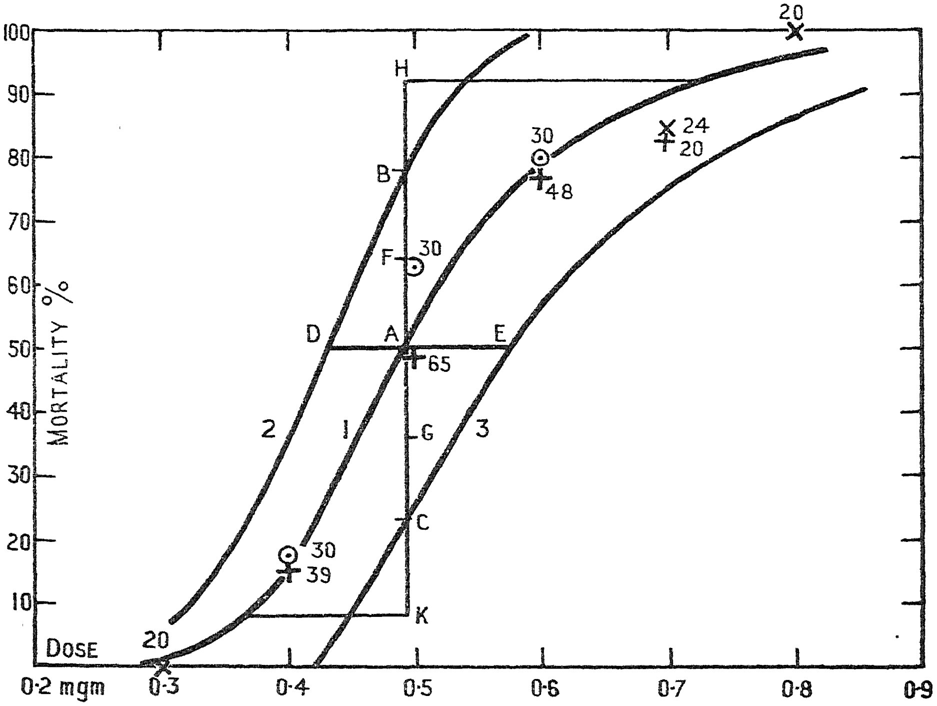

FIG. 2.—Characteristic for cocaine (A) from fig. 1, by intravenous injections into mice. Observed points 0 − 0 obtained six months later than + − + and × − × twelve months later. The curves through E and D represent limits of the mortality which any dose may be expected to give in 369 cases out of 370, when injected into 30 mice. KH represents the same limits for 0·505 mgm. using only 10 mice, and GF the limit using 120 mice. DE represents the limits of the doses that may give 50 per cent. mortality in 30 mice.

bell-shaped frequency curve can be approximately determined by plotting the differences in percentage mortality for successive small dose intervals against the respective doses as abscissæ. The differences have been measured for the smooth curve drawn through the observed points for the killing of mice by cocaine hydrochloride (fig. 2, middle curve). The figures obtained are tabulated in Table II. The increment in percentage mortality is given in the second column of this table against the corresponding interval of dose given in the first column. Fig. 3 is constructed by plotting the numbers in column 4 against the mid-points of the dose-intervals to which they correspond. The frequency distribution, as will be seen, is asymmetrical.

Table II.—Frequency distribution of individual lethal doses of cocaine for mice, from smoothed curve, fig. 2.

Dose Interval.	Mid-point of dose Interval (d).	Difference of Percentage Mortality (f).	$f \times d$.
0·2 – 0·3	0·25	1	0·25
0·3 – 0·4	0·35	15	5·25
0·4 – 0·5	0·45	38	17·11
0·5 – 0·6	0·55	25	13·76
0·6 – 0·7	0·65	11	7·15
0·7 – 0·8	0·75	6·5	4·88
0·8 – 0·9	0·85	2·5	2·125
0·9 – 1·0	0·95	1·0	0·95
		S (f) 100	S (fd) 51·475

A.—Arithmetical mean $= \frac{S(f \times d)}{S(f)} = 0{\cdot}515.$

B.—Median $= 0{\cdot}4895.$

C.—Mode $= A - 3\ (A - B) = 0{\cdot}439.$

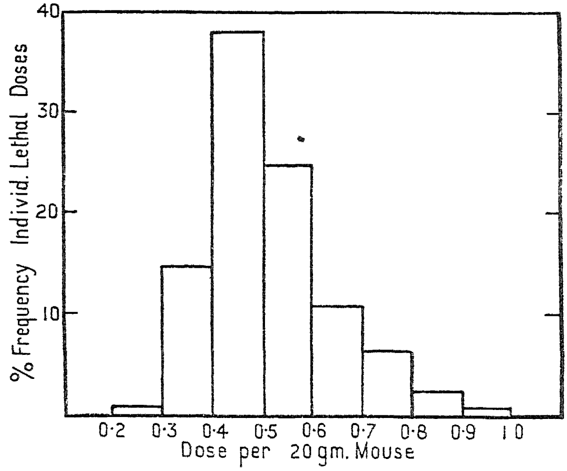

Fig. 3.—Distribution of individual lethal doses for cocaine when injected intravenously into mice; derived from the smoothed curve of fig. 2.

Animals with individual lethal doses between 0·45 and 0·5 mgm. per 20 gram body weight occur more frequently than animals with individual lethal doses in any other dose interval, *i.e.*, the "mode" of the frequency distribution is between 0·45 and 0·5 mgm. The frequency of the occurrence of animals with higher individual lethal doses than these falls off less rapidly than that of animals with lower individual lethal doses. This type of asymmetry, with the mode on the side of the smaller doses, seems to be the commonest amongst the poisons so far studied. The greatest asymmetry yet observed is that of the curve for the killing of mice by dysentery toxin; for many drugs it is of the same order as that shown for the lethal action of cocaine hydrochloride on mice, and the dispersion of the distribution is not very widely different. For the action of digitalis on frogs it is almost symmetrical.

We may return now to the consideration of the particular dose in this range which affords the best estimate of the toxicity of a drug, taking the lethal action of cocaine hydrochloride as an instance. The logical method is to take, as an index, the toxicity of the drug for the average animal of the species. There are three different values which can be taken as expressing the "average" of a series of figures such as these:—(1) The arithmetic mean of all individual doses. (2) The median, *i.e.*, the particular value of the individual lethal dose which divides all animals of the variety used into two groups of equal size, and which when injected into an exceedingly large group would therefore kill exactly 50 per cent. (3) The mode—already referred to above, the individual lethal dose which occurs most frequently, and which is the dose at which the inflexion, or steepest portion, occurs in the S-shaped mortality curve of fig. 1.

The arithmetic mean can be calculated approximately from Table II, by multiplying the mean of the figures of column 2 by the figures in column 3, adding the products so obtained (set out in column 4), and dividing this sum by the sum of column 3, which is, of course, 100. The mean individual lethal dose of cocaine for mice so obtained is 0·515 mgm. The median is obtained by reading off from the smoothed mortality curve the dose necessary to kill 50 per cent. It is 0·490 mgm. It may be obtained also by the following method. The sum of the differences of percentage mortality (Table 2) from 0·2 to 0·4 is 16. The median is therefore a dose which is between 0·4 and 0·5 mgm. and is greater than 0·4 by $\frac{50-16}{38} \times 0{\cdot}1$, 38 being the percentage difference of mortality between doses of 0·4 and 0·5, and 0·1 being the dose interval between 0·4 and 0·5. This works out at 0·0895, so that the median individual lethal dose is 0·4895. The mode, as already pointed out, is between 0·4 and 0·5 mgm. and is approximately obtained by the following formula (Yule):—

$$\text{Mode} = \text{Mean} - 3\,(\text{Mean} - \text{Median}).$$

Calculated in this way for cocaine it is equal to 0·439 mgm. It is the point of the maximum slope of the characteristic. The mean can also be calculated in a similar manner from the actual observed percentage mortalities (Column 4 in Table I) by treating the differences in these percentages as above. The mean lethal dose so calculated is 0·516 mgm., the median 0·486 mgm. and the mode 0·426 mgm.

For the degrees of asymmetry which are usual in dealing with most poisons (the case for dysentery toxin is rather exceptional), the median differs from the arithmetic mean by an amount which is within the error of determination, when small numbers of animals are used. So that, although the arithmetic mean is theoretically the best representative of a series of variables, no great disadvantage is incurred by using the median of the individual lethal doses to fix a toxicity. It has the advantage also over the mode and the arithmetic mean, in that the whole mortality curve need not be mapped out for its determination ; all animals available can be injected in groups with doses around the median, the median being approached by trial in successive groups until the dose which kills 50 per cent. is "bracketed."

I would suggest that the term "minimal lethal dose," with its variable meaning, should be dropped altogether, and that toxicity should be stated primarily in terms of the "median lethal dose," that is the dose which kills 50 per cent. of a large group of animals. As a convenient abbreviation I would suggest for this the symbol LD 50, which will be used in the following discussion. For doses which kill other proportions of large groups of animals it is convenient to use the analogous symbols LD 75, LD 25, for doses which kill 75 per cent., 25 per cent., and similarly for doses killing other proportions. There are other reasons, discussed later, of a statistical nature, for preferring the use of a dose in the neighbourhood of the average lethal dose.

II. *The Accuracy of Determination of the Lethal Dose.*

It is generally admitted that determinations of toxicity require the use of large numbers of animals, if any degree of accuracy is to be obtained, but very few attempts have been made to determine the relation between the numbers of animals injected and the possible error. If there were no differences between the individual lethal doses, the final fixation of the toxic dose would depend on the injection of two animals only, one with a dose x, and another with a dose $x + \delta x$, where δx is a small fraction of x, and repetition of the experiment would always give the same result, within the limits of error of measurement of the dose and the weights of the animals. The conditions encountered in practice are quite different. Suppose two doses of cocaine hydrochloride,

x and $x + \delta x$, where δx has the same significance as above, are injected into a pair of mice. Then if the dose x fails to kill, and the dose $x + x\delta$ kills, it will be found that the value of x, on different pairs of mice, varies from about 0·3 to about 0·9 mgm. Further, it will occasionally happen that the dose x will kill, and the dose $x + \delta x$ will not kill. Nor can it be said that, if both animals die, the dose is greater than the certainly lethal dose, which would be true for the ideal case discussed above.

These discrepancies arise, of course, from the fact that in an assembly of mice with different individual lethal doses, it is a matter of chance which particular mouse is chosen for injection. For instance, there is approximately one chance in ten that a mouse injected with a dose of 0·38 mgm. will die, and the chance that a dose will kill does not become negligible until the dose is reduced to about 0·28 mgm. At the other end of the scale, the chance of survival does not become negligible until the dose reaches 0·85 mgm. to 0·9 mgm.

On the other hand, if sufficiently large groups of animals are injected at each dose, increasing the dose will always give rise to increasing mortality, and the observed mortalities will fall more and more closely on a smooth mortality curve (such as those shown in figs. 1 and 2) as the size of the groups increases. If an attempt is made to measure the size of a dose of cocaine by its toxicity, with a pair of mice, the death of one of these, together with the survival of the other, will only indicate that the dose of cocaine injected is somewhere between 0·3 and 0·9 mgm., whereas when a very large group of animals is injected, the percentage mortality amongst them will indicate the exact size of the dose, to within a few units per cent.

The problems which present themselves at this point are: (1) What is the size of the animal group which must be used to attain any given degree of accuracy? and (2) What particular dose, in the whole range of the mortality curve, can be determined with the least expenditure of animals? Let us begin with the median lethal dose, LD 50 as defined above, which is 0·490 mgm. for cocaine on mice, and make the assumption that the smoothed curve represents the curve which would be obtained with indefinitely large groups. If this dose is injected into groups of, say, 30 mice, the number that die in each group will vary. It will only occasionally happen that exactly fifty per cent. will die. On the other hand, the chance that the whole of a group of 30 will survive, or that the whole of a group of 30 will die, after the injection of that dose, is so small that it can be neglected, provided that the group of mice has been chosen at random. The actual range of the number of deaths to be expected can be calculated from the well-known formula for the standard deviation:—

$$\text{Standard deviation} = \sqrt{pq\text{N}}$$

where p is the probability of death, q that of survival (both p and q in this case being 0·5), and N the number of animals used. This works out at 2·74, and the probability is approximately 0·9973 (say 370 to 1) that, in any group of 30 injected with a dose of 0·490 mgm., not more than $15 + 3 \times 2{\cdot}74$, and not less than $15 - 3 \times 2{\cdot}74$ animals will die. If 371 groups of 30 are injected, it is probable that only one group will show a number of deaths outside the limits $15 \pm 8{\cdot}22$, or, expressed as a percentage, $50 \pm 27{\cdot}4$. This probability is large enough to be taken, and is usually taken for practical purposes, as certainty. About 19/20 of the observed number of deaths will lie between the limits $15 \pm 5{\cdot}48$, and half of them will lie between the limits $15 \pm 1{\cdot}823$ ($= 2/3$ standard deviation). The limits corresponding to three times the standard deviation are plotted on the diagram in fig. 2 at B and C, AB being equal to 27·4 per cent.

Similar deviations have been worked out for the percentage mortalities from about 5 per cent. to about 95 per cent., substituting for p and q the respective proportions corresponding to each mortality. The points so obtained have been plotted on fig. 2, and the smooth curves 2 and 3 drawn through them. These curves are very rough at the extremities for the following reasons. For the small percentage death-rates (or survival rates) at the ends of the curves the deviation of mortality becomes very asymmetrical, deviations in the direction of larger death rates being more frequent at the lower end of the curve, and in the direction of smaller death rates being more frequent at the upper end. They can, however, be used to determine the errors, at least to a first approximation. These curves can be used to determine the accuracy with which the size of a dose of cocaine hydrochloride can be measured by its toxic effect on a single group of 30 mice. Such an operation is, of course, of little practical importance in dealing with cocaine, but this drug is used for illustrative purposes, and the results apply, with appropriate modification of scale, to all determinations of lethal dose, whatever drug is used. Cocaine is chosen as an example because it is obtainable in a stable crystalline form.

If 30 mice were injected with a dose of cocaine of unknown size, and 15 died, it is clear that of all possible dimensions the dose might have, 0·490 mgm. would be the most likely. But it is also clear from what has been said above, that the chances are small that it was exactly 0·490 mgm. Accepting the probability 0·9973 as practical certainty, it might be as low as 0·430, the point D on the diagram in fig. 2 being the upper limit of the mortality deviation for this dose, or it might be as high as 0·576 mgm., the point E on the diagram corresponding to the lower limit of the deviation for this dose. The range of possible doses which might give rise to a mortality of 50 per cent., represented as a percentage of the middle dose of the range, is

$$\frac{0\cdot 576 - 0\cdot 430}{0\cdot 490} \times 100 = 29\cdot 8 \text{ per cent., or approximately } \pm 15 \text{ per cent.}$$

More accurately, the positive error is $\frac{0\cdot 576 - 0\cdot 490}{0\cdot 490} \times 100 = 17\cdot 5$, and the negative error $\frac{0\cdot 490 - 0\cdot 430}{0\cdot 490} \times 100 = 12\cdot 2$ per cent.

The ratio of the highest dose which will give a mortality of 50 per cent. to the lowest dose which will give that mortality is $\frac{0\cdot 578}{0\cdot 430} = 1\cdot 34$.

By drawing horizontal lines in this manner through the curves at mortalities of 10 per cent., 20 per cent., 30 per cent., etc., the errors associated with the determination of the value of doses giving these mortalities have been estimated. The results are given in Table III. The last column gives the ratio of the highest and lowest doses for each mortality rate. It will be seen that the range of dose deviation, as shown by this ratio, is smallest for a mortality rate of 40 per cent. and is practically constant from 20 to 60 per cent. For more symmetrical curves this range will extend to higher mortality rates. The deviation becomes indefinitely large at the ends of the curves. Therefore the

Table III.—Dose errors for cocaine hydrochloride at different mortality rates in a group of 30 mice.

No. of Animals dying out of 30.	Percentage Mortality Rate.	Percentage Error of Dose.		Ratio of Highest to Lowest Dose giving each Mortality Rate.
		Positive.	Negative.	
0	0	66·8	100	0·0
1	3·3̇	30·3	10·6	1·48
3	10	21·3	13·3	1·405
6	20	17·5	12·2	1·34
9	30	16·1	12·5	1·320
12	40	16·8	11·4	1·318
15	50	17·75	12·2	1·34
18	60	19·3	12·6	1·36
21	70	22·1	14·3	1·415
24	80	23·1	18·15	1·51
27	90	20·1	23·75	1·58
29	96·6̇	11·2	29·6	1·60
30	100	Indefinite.	66·6̇	>1·6

median lethal dose has further advantages over those already stated, in that it is in the neighbourhood of doses with the minimum relative error of determination, whereas the "certainly lethal dose" and the "maximum tolerated dose" have indefinitely large errors. The indefiniteness of the error at the ends of the characteristic is clearly due to the fact that, if either all die or all survive of a group of animals injected with an unknown dose of the drug, the information supplied by the event is merely that the dose is, in the one case, indeterminately greater than that represented by a certain point on the characteristic, or, in the other case, indeterminately less than that represented by another point, and the actual magnitude of these limiting doses depends on the number of animals used.

The use of larger or smaller groups of animals will, of course, lead to smaller or larger errors. Fig. 4 gives the deviation of mortality to be expected in different sized groups of animals, when injected with the median lethal dose of *any* drug; the deviation depending only on the chance of death or survival.

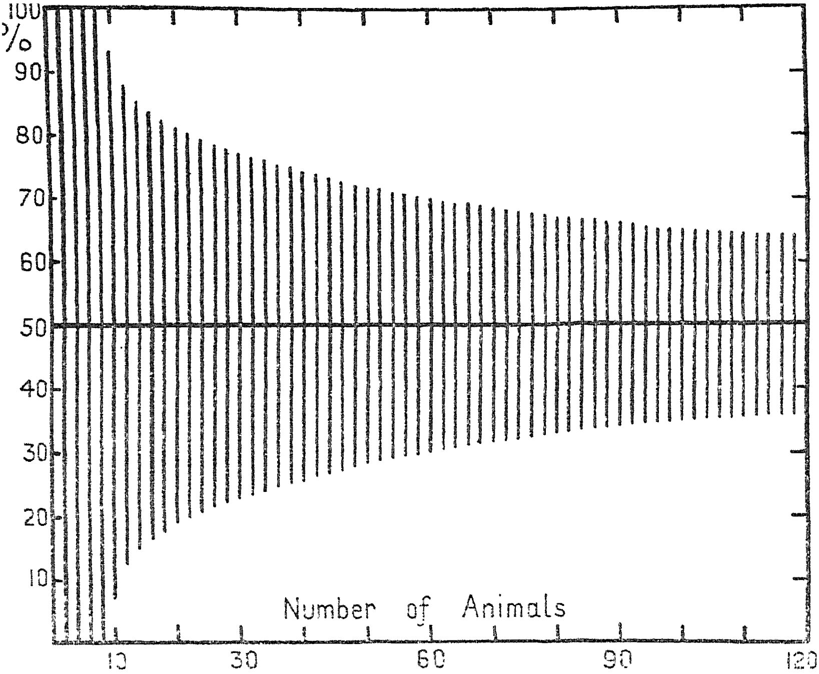

Fig. 4.—Limits of percentage mortality obtainable by chance when the average lethal dose causing 50 per cent. mortality is injected into different sized groups of animals. The limits are represented by the lines as percentage of the number of animals used, and correspond to ± three times the standard deviation of the mortality.

The figures are obtained by substituting the requisite number for N in the formula for three times the standard deviation, for groups larger than 20. The deviations for values of N below 20 are obtained from the terms of the binomial expansion $(0\cdot5 + 0\cdot5)^N$. The deviation plus or minus is half the number of those terms (symmetrically disposed about the middle term), of which the sum is 0·9973 of the sum of all the terms of the series. For values of N = 20 the number of these terms practically coincides with $6\sqrt{pqN}$. The ordinates are percentage mortality, the abscissæ the number of animals in a group injected with the average lethal dose. The theoretical mortality is the same for every group number—50 per cent. The black lines, with their mid-points at 50 per cent., represent the deviations for different group numbers of animals, expressed as percentage of the group number. The percentage mortality in any group of animals, resulting from the injection of LD 50, may therefore be expected to fall somewhere on the black line corresponding to the number of animals in the group. Thus the percentage mortality to be expected if 5 animals are used may be between 0 per cent. and 100 per cent., if 10 animals, on an average 4 per cent. to 96 per cent., if 120 animals, 36 per cent. to 64 per cent., and so on; the length of the black lines diminishing as the square root of the number of animals.

The percentage error of measurement of *dose* caused by these deviations is less, and will, of course, depend on the slope and shape of the characteristic. Not only so, but the error in dose diminishes with increasing size of group more rapidly than the deviation in mortality when the number of animals used is less than about 30. This is illustrated in fig. 2, where the deviations of mortality for groups of 10 (HK), 30 (BC), and 120 (FG) animals are plotted.

The deviations of dose corresponding to these deviations of mortality are obtained by drawing horizontal lines at the ends of the mortality deviation. The points where these lines cut the characteristic give the range of dose deviation for each size of group of animals. It will be seen that the deviations for 120 and 30 animals fall wholly on the part of the characteristic which is practically a straight line, whereas that for 10 falls outside the straight portion of the characteristic. The dose error thus calculated differs from that on p. 493 in that this gives the range in dose error for a fixed dose, in this case the average lethal dose, whereas the dose error on p. 493 is that corresponding to a fixed mortality. The dose error for a fixed mortality will increase in a similar manner but not quite to the same extent.

In consequence, the increase in error of the average lethal dose, produced by diminishing the size of animal group from 120 to 30, is proportional to the percentage mortality deviation plotted in fig. 5; *i.e.*, usually proportional to the square root of the number of animals used. Below this, the dose error

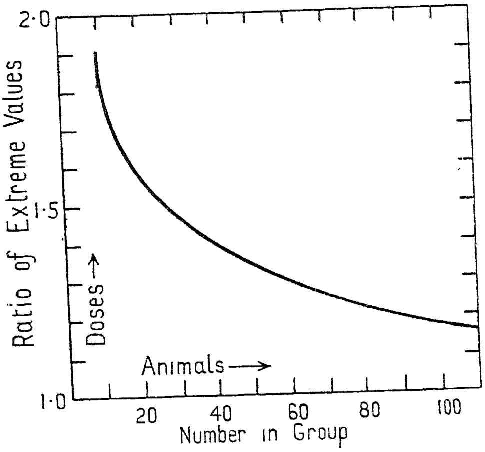

Fig. 5.—Ratio of extreme values assigned to an average lethal dose of cocaine by the use of different sized groups of mice.

increases more rapidly than the mortality errors given in fig. 4 because of the increasing curvature of the characteristic.

An important practical point emerges from these considerations. A group of about 30 animals is near the optimum size for the determination of an average lethal dose. A significant increase of accuracy above that given by this group number is only obtained by the use of greatly increasing numbers of animals; whilst diminution of the group number below 30 leads to a very rapidly increasing error. The ratio of the cost of the test to the accuracy obtained reaches a minimum in the neighbourhood of this group number. This applies to all those drugs so far tested.

The relatively rapid increase in dose error with animal groups of a size less than 20 to 30 is shown in fig. 5. The points on the curve give the ratios of the extreme values which might be assigned to a median lethal dose if the value of the dose were estimated by reading from the characteristic the dose corresponding to the mortalities actually obtained with such groups. This ratio becomes indefinitely large when the group number diminishes below 10.

A simple formula will give an approximation to the error of determination of doses in the neighbourhood of the median lethal dose, once the slope of the "straight" part of the characteristic is obtained. By determining the LD 25 and the LD 75 on a fairly large number of animals, this slope is obtained with sufficient accuracy for most drugs.

Let N be the number of animals injected with a dose near the LD 50, D the deviation of dose (reckoned as a percentage of the dose) corresponding to three times the standard deviation of the mortality, K the ratio of the percentage increase of dose to percentage increase in mortality, and p the probability of survival, and q that of death, with the dose injected, p and q being 0·5 for the average lethal dose.

Then let three times the standard deviation of deaths $= d$.

$$d = 3\sqrt{pq\mathrm{N}}.$$

Let d be reckoned as percentage of $\mathrm{N} = d'$.

$$d' = \frac{3\sqrt{pq\mathrm{N}}}{\mathrm{N}} \times 100.$$

Since $\mathrm{D}/d' = \mathrm{K}$, for parts of the curve which approximate to straight lines, then

$$\frac{\mathrm{D\,N}}{300\sqrt{pq\mathrm{N}}} = \mathrm{K}. \qquad \frac{\mathrm{D}\sqrt{\mathrm{N}}}{300\sqrt{pq}} = \mathrm{K}.$$

$$\sqrt{\mathrm{N}} = \frac{300\ \mathrm{K}\sqrt{pq}}{\mathrm{D}}$$

$$\mathrm{N} = \frac{90{,}000\ \mathrm{K}^2\,pq}{\mathrm{D}^2}. \qquad (1)$$

For cocaine, the value, for the median lethal dose, of K is 0·510 and for N = 30, the error of the average lethal dose is

$$\mathrm{D}^2 = \frac{90{,}000 \times (0{\cdot}510)^2 \times 0{\cdot}5 \times 0{\cdot}5}{30},$$

$$\mathrm{D} = \pm\,14{\cdot}05,$$

as against + 17·75 or — 12·2, as worked out by the graphic method (p. 493). The formula applies to values of D not larger than about 20 per cent., provided that only the "straight" part of the characteristic is involved in the calculation. For very small values (up to 5 per cent.) of D, it is applicable to any part of the curve—the value of K being the ratio of the percentage increment of dose at the point under investigation to the percentage increment in mortality.

The estimate of the accuracy of the determination of the toxicity clearly depends on the liability of casual groups of animals of sufficient size to give characteristics of the same shape, and average lethal doses of the same magnitude. Observations on the toxicity of cocaine over a period of more than twelve months have shown that at different intervals the percentage mortality for any given dose does not vary significantly from that given in the curve.

The series of points marked with vertical crosses in fig. 2 were obtained about six months before those marked with circles, and those marked with diagonal crosses about twelve months later, and the difference between these two series is well within the significant limits. So that, in experiments demanding the knowledge of the toxicity of cocaine (as, for example, in the exploration of possible antidotes for cocaine poisoning) the expected mortality for any given dose can be read from the curve, for casual samples of mice, not specially selected. The injections must be made in a similar manner to those used for the determination of the curve, namely :—

(1) The dose must be proportional to the weight of the animal, and be injected intravenously.

(2) The concentration of the solution injected must be such that 0·5 c.c. contains 0·5 mgm.

(3) Variations of dose, whether necessitated by the weight of the animal, or made for the purpose of obtaining different points of the curve, must be obtained by altering the total volume of solution injected.

Unless these conditions are satisfied, the characteristic given will not represent the relation between dose and mortality. The evidence for this will be given in a further paper on the toxicity of cocaine and certain of its derivatives.

III. *Periodic Variation of Median Lethal Dose.*

Unfortunately, however, several substances for which only biological methods of standardisation are available, do not give the same median lethal dose when large groups of animals are injected on different days. Some figures representing the difference in the median lethal dose as determined in the summer and

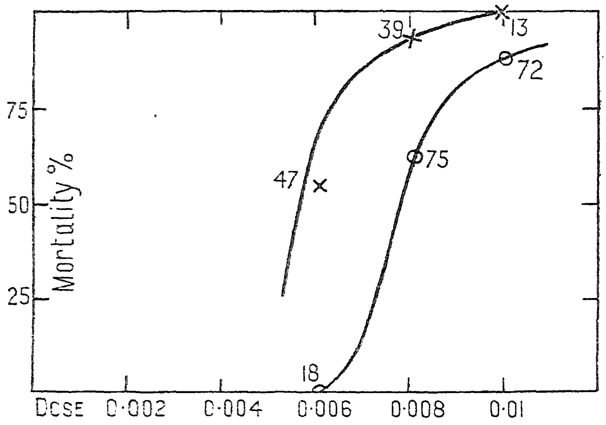

Fig. 6.—Diphtheria toxin characteristics for guinea-pigs in summer and winter (Sudmersen and Glenny).

in the winter, for diphtheria toxin, taken from a paper by Glenny and Sudmersen, are plotted in fig. 6. No alteration in composition of the toxin can account for these differences, for the summer curve is made up of two sets of figures obtained in two succeeding summers with the same toxin, the results agreeing in the two summers. Similar, but more rapid periodic variations occur in standardising the digitalis series by the injection of frogs. I have had most experience of this type of variation in the standardisation of insulin by determination of the convulsive dose in mice. The method and results are given in a contribution to a report to the Health Committee of the League of Nations (Trevan and Boock, 1926). Two characteristics out of five, obtained with the same sample of insulin on different dates, are shown in fig. 7, curves A and B. As will be seen, the figures obtained on different occasions varied; in series B the median convulsive dose was 0·00155, whereas in the others, using about 900 mice, it was practically 0·00113.

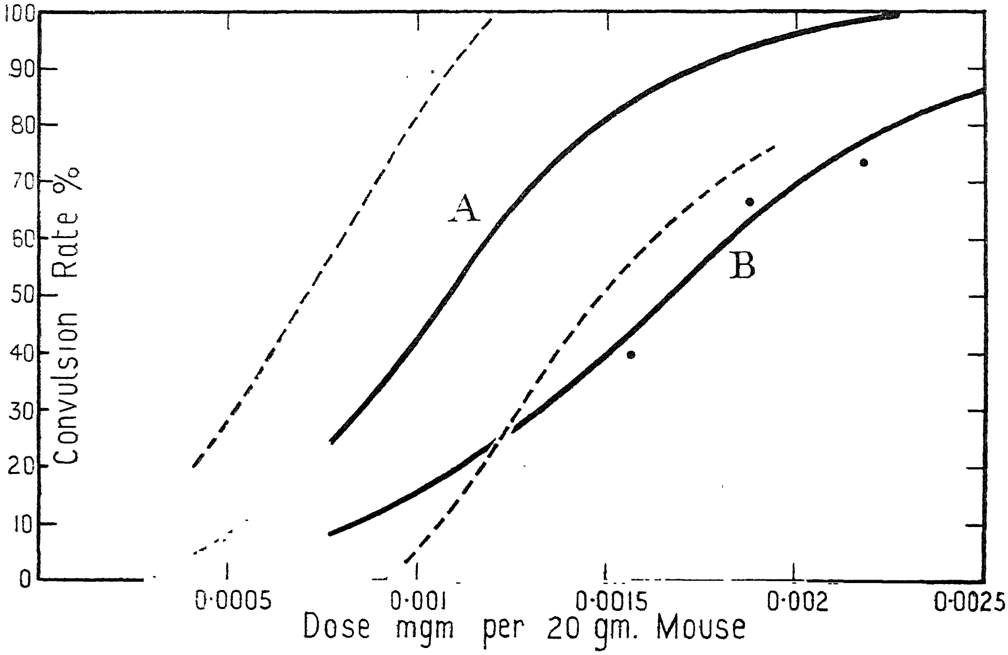

FIG. 7.—Insulin characteristics for mice on different days, A and B, and limits of possible errors when a "test" sample and a "standard" sample are injected into two groups of 30 mice each.

In series B it will be seen that each of the results for groups of 30 animals lies outside the dotted line, which represents the limits of variation from the first curve for groups of 30 animals if the variation is solely due to errors of sampling.

(See later.) The displacement of curve B is therefore largely due to a decrease in susceptibility of the whole stock of mice to insulin. Variations of this order of magnitude are uncommon, and have, so far, not been traced to any specific factor. Similar variations in susceptibility to insulin have been described by Marks (1926) as occurring amongst rabbits. But even for the extreme case shown in curve B, although the median convulsive dose is 54 per cent. greater, the distribution of the individual convulsive doses around the mean is similar to the distribution on the days of greater susceptibility; so that, for example, the dose causing 30 per cent. of mice to convulse is also 54 per cent. greater, within the error due to the practical limitation of the size of the groups injected. This is shown in the figure, where the line B is constructed by increasing the abscissæ of the smoothed curve A by 54 per cent. It will be seen that the actual figures obtained, represented by the dots, do not deviate by a significant amount from the smoothed curve B. The ratio of percentage increment of dose to percentage increment of mortality for corresponding points is the same, whatever the median convulsive dose. Curves of the same shape are given by samples prepared in different ways. Since the median convulsive dose varies in this fashion, the curve cannot be used for the absolute determination of the value of an unknown sample in the manner described for cocaine. Any of these curves can, however, be used for the comparison of the activities of two samples, in one of the following ways :—

(1) The two samples can be injected over a wide range of doses, so that two characteristics, one for each sample, are obtained. The ratio of the abscissæ of the two characteristics gives the ratio of activities.

(2) The two samples can be injected into large groups of animals, at doses which by preliminary experiments are adjusted to give about 50 per cent. of convulsions for each sample. The convulsion rate with each sample is noted and the doses which gave these rates read off from one of the convulsion-dose curves, such as either of those in fig. 7. The ratio of these doses then gives the ratio of potency of the doses actually injected.

The error of such a determination, with a given number of animals, will be larger than that given for determinations of toxicity when no variation of average dose occurs. There will be a statistical error affecting the group injected with the standard preparation, as well as one affecting the group injected with the unknown. The standard deviation of the difference between the two mortalities obtained, expressed as a percentage, is given by the formula,

$$= 100 \sqrt{pq\left(\frac{1}{m} + \frac{1}{n}\right)}$$

where n is the number of animals in the group injected with the standard, m that of the group injected with the unknown, p and q the probabilities of death or survival (or occurrence or non-occurrence of convulsions, for insulin) with the standard dose.

It is easy to show algebraically that, for a given number of animals 2N, the standard deviation is a minimum when $m = n = \mathrm{N}$. The number of controls injected with the standard should therefore be equal to the number injected with the unknown, and the standard deviation of the difference between the two mortalities obtained becomes :—

$$100 \sqrt{2pq/\mathrm{N}},$$

where N is the number of animals in each of the comparative groups.

The formula becomes then

$$\mathrm{M} = 180{,}000\ \mathrm{K}^2 pq/\mathrm{D}^2$$

(where D is the dose error corresponding to three times the standard deviation of the mortality).

For insulin, $\mathrm{K} = 0{\cdot}967$, and for $\mathrm{D} = 10$, and $p = q$ (median convulsive dose),

$$\mathrm{N} = \frac{180{,}000 \times (0{\cdot}967)^2 \times \frac{1}{4}}{100} = 451,$$

and for $\mathrm{N} = 30$, $\mathrm{D} = 37{\cdot}4$ per cent.

The deviations at different mortalities are given by the dotted curves for curve A, and give similar figures for the error.

The results of the injection of 27,000 mice with batches of insulin have been analysed to see how far the prediction of the frequency of deviations of a given magnitude are fulfilled. Table IV is the result of the examination of twelve of these batches. In the second column is tabulated the number of groups of 60 mice used for testing each batch, thirty mice in each group being injected with the batch under test and thirty with a standard preparation (Trevan and Boock, 1926). The convulsion rates were noted for the batch under test and the standard in each group of 60, and an estimate of the relative activity of the two samples obtained in the following manner. The doses are read which correspond to these convulsion rates on curves A or B (fig. 7)—either will give the same ratio of activities. The ratio of the doses so read from the curve is taken as the ratio of the activities of the doses actually injected, and by appropriate allowance for the dilutions injected, a value for unit quantity of the batch under test in terms of unit quantity of the standard insulin is obtained. The

Table IV.—Standard deviations of a series of insulin estimations, calculated by the mean square method from the observed deviations for each batch.

Batch.	Number of sets of 60 mice used.	Standard deviation of one observation as per cent. of mean.
1	27	14·3
2	13	13·04
3	18	14·8
4	23	12·93
5	20	11·78
6	12	12·17
7	14	12·23
8	18	10·35
9	21	13·18
10	20	12·51
11	27	13·85
12	17	10·73
		Average 12·65
		Calculated from shape of curve, as described in text = 12·45.

mean of these values is calculated for all the groups of sixty mice used on a batch, and the standard deviation for the result from a single group worked out for each batch by the method of mean squares. These standard deviations are given in column 3 as percentages of the corresponding mean. Since the convulsion rates in all these groups are in the neighbourhood of 50 per cent., the standard deviation so obtained should be in the neighbourhood of $\frac{1}{3}$D = 12·45, assuming the mean value obtained for each batch is sufficiently near the true value of the batch. The actual values do not depart by significant amounts from this value.

The figures therefore are in accord with the initial assumptions, that the conditions of random sampling have been observed for the mice used, and that the method of calculating the distribution of the errors from the characteristic is a sufficiently close approximation for practical purposes. The same point may be presented in a different form. For a series of 455 tests on groups of 60 mice, including those given above and others, the frequency of the occurrence of deviations corresponds closely with what would be expected in a normal distribution with a standard deviation of 12·45 per cent. of the mean. The figures are given in Table V. The calculated frequencies are obtained from a table of the normal distribution in terms of a standard deviation of 12·45 per cent. (*cf.* Fisher, p. 76). The frequency of errors not exceeding ± 37·4 per cent.

(= D) is 454 out of 455. The calculated frequency is 453·8. 436 times the error did not exceed ± 25 per cent. The calculated value is 435. 274 did not exceed ± 10 per cent. The calculated number is 263.

Table V.—Assay of insulin. Comparison of observed frequency of deviations with frequencies expected if the distribution were normal with a standard deviation of 12·45 per cent.

Percentage Deviation from Means of each Batch.	Frequency of Negative Deviations.		Frequency of Positive Deviations.	
	Observed.	Calculated.	Observed.	Calculated.
0 to 5	90	71·5	63	71·5
5 to 10	64	60	57	60
10 to 15	46	44·25	34	44·25
15 to 20	33	27·25	20	27·25
20 to 25	11	19·5	16	19·5
25 to 30	4	6·3	8	6·3
30 to 37·4	1·0	3·1	7	3·1
>37·4 (Actual value 42)	0	0·6	1	0·6

The figures are plotted in fig. 8. The dotted lines represent the normal distribution for a standard deviation of ± 12·45 per cent. and the full lines the observed frequencies.

Inspection of this figure suggests that the distribution actually obtained is not quite normal. There is an excess of values between 0 and — 5 per cent., a deficit for extreme values in the negative direction and an excess of extreme positive values. These facts suggest that the true form of the distribution is slightly skew, with the mode on the negative side of the mean between 0 and — 5 per cent. I have tested the significance of the variation from the normal distribution by calculating χ^2 (Fisher, Cap. 4) for the figures in Table V. The value of χ^2 after combining the frequencies of deviations above + 30 per cent. and also those below — 30 per cent. works out at 24·224; P is therefore 0·03, taking n as 13. A larger value of χ^2 would only occur about once in 35 such sets of 455 tests if the distribution were normal. The difference between the normal distribution and that obtained is probably therefore just significant. That the distribution is not quite normal depends in the end on the asymmetry of the insulin characteristic. The dose errors for a convulsion rate of 50 per cent. obtained from the horizontal distances between the characteristic and the dotted lines (fig. 7) are + 37·6 per cent. — 34·4 per cent., an asymmetry in the same direction as and of a magnitude similar to that obtained. The deviation from the normal distribution is, however, not large enough to be of practical importance.

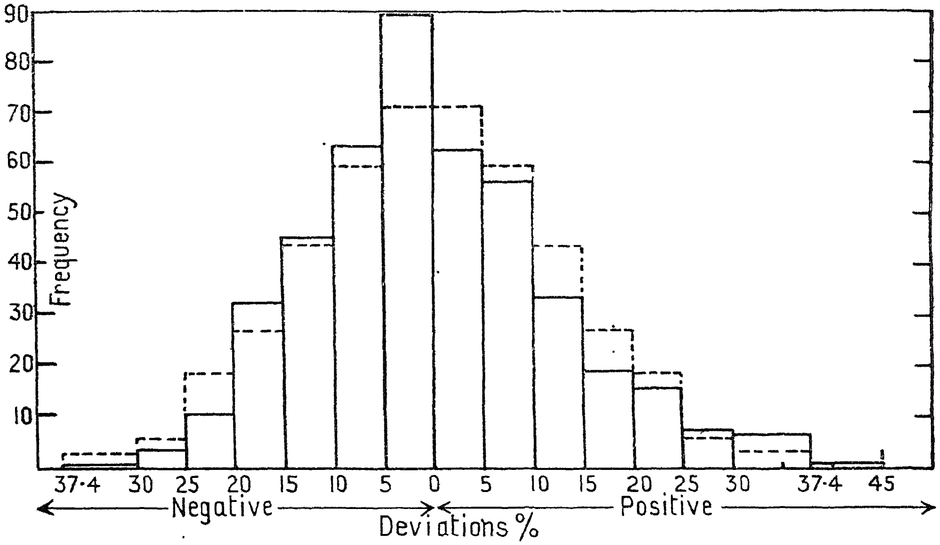

Fig. 8.—Frequency of deviations from mean value of estimation of value of insulin solutions on 450 groups of 60 mice, half injected with a "standard" insulin, and half with a "test" solution. The dotted lines represent a normal frequency curve of the same area, and with a standard deviation, corresponding to that calculated from the characteristic as explained in the text (12·6 per cent.). Some of these results are tabulated in Table V.

For diphtheria toxin, the value of K for the median lethal dose is 0·323. So at that dose the number of animals to be used, when the toxicities of two samples are compared with one another, is, for an error of ± 10 per cent. :—

$$N = \frac{180{,}000 \times (0\cdot323)^2 \times \frac{1}{4}}{100}$$

$$= 46\cdot8 \text{ in each group.}$$

For a 20 per cent. error, 11 to 12 animals in each group would suffice. The number of animals represented in fig. 6 render this calculation only very rough, but the order of accuracy attainable with the titration of diphtheria toxin on guinea-pigs is very much greater than that so far attained with the titration of insulin on mice.

The titration of diphtheria *antitoxin* against a "standard" toxin is considerably more accurate than that of toxin alone, for the following reasons. Antitoxin is measured by its neutralising power for toxin. Quantities of about 100 "lethal" doses of toxin are injected into each guinea-pig used, but mixed before injection with a quantity of antitoxin which is varied for different animals until two mixtures are found, one of which kills, and one, with a slightly larger amount of antitoxin, fails to kill. The mixture which has only just not sufficient antitoxin to save the animals, say x c.c., is taken as containing about one lethal dose of toxin unneutralised, and the amount of antitoxin in it is considered to be sufficient to neutralise 99 lethal doses of toxin. The accuracy of this figure as a representative of the real number of lethal doses absorbed by x c.c. of the antitoxin depends on the number of animals injected with the mixture containing 1 lethal dose in excess. As already stated, 24 animals injected, 12 with the unknown and 12 with the standard, will determine a median lethal dose with an accuracy of $\pm$ 20 per cent. Under such conditions a mixture may be said to contain 1 lethal dose when it really contains as little as 0·8 or as much as 1·2, and the real equivalent in toxin of x c.c. of antitoxin is therefore between 98·8 and 99·2 lethal doses. If the neutralisation of toxin by antitoxin followed the laws of simple chemical proportion, these figures would represent the accuracy with which the amount of antitoxin present in a sample of serum could be titrated. Actually, the relationship between number of lethal doses absorbed and amount of serum added to a toxin is such that progressively more antitoxin is required to neutralise one lethal dose as complete neutralisation is approached. (Glenny, Pope and Waddington, 1925.) With a fresh toxin of small average lethal dose, the effect of this is to multiply the error calculated above by about 10 times, so that the final value of the serum in antitoxic units (1 antitoxic unit being equivalent to 100 MLDs.), will be 99 $\pm$ 2·0—an error of 2 per cent. For such degrees of accuracy, the toxin used should contain as many lethal doses as possible in 1 c.c., and as little toxoid, the degree of accuracy being proportional to the number of lethal doses.

The relatively high degree of accuracy of titration of antitoxin by this method has probably led, in the past, to some exaggeration in estimates of the accuracy of biological standardisation in general. It is clear that all antitoxic sera should be titrated by the determination of the amount necessary to protect an animal against the largest number of lethal doses it is possible to inject.

IV. *Error from using Limited Numbers of Animals.*

The practical problem as to what results are to be expected when the number of animals used is below 10, deserves special consideration. It is easy, though laborious, to work out from the terms of a binomial expansion the chances for any number of animals. Table VI gives the chances of distinguishing between

Table VI.—Limits of accuracy with 10 mice in two groups, one injected with LD 25, and the other with LD 75.

Possible results. Mortality out of 5.			Probability of Occurrence.
A.—Experiments in which the doses will be placed in right order.			
Dose LD 25		LD 75.	
0/5	and	5/5	0·9215
	or	4/5	
	or	3/5	
	or	2/5	
	or	1/5	
1/5	and	5/5	
	or	4/5	
	or	3/5	
	or	2/5	
2/5	and	5/5	
	or	4/5	
	or	3/5	
3/5	and	5/5	
	or	4/5	
4/5	and	5/5	
B.—Experiments in which doses will be called equal.			
1/5	and	1/5	0·0583
2/5	and	2/5	
3/5	and	3/5	
4/5	and	4/5	
C.—Experiments in which LD 25 will be called greater than LD 75			
5/5	and	4/5	0·0197
	or	3/5	
	or	2/5	
	or	1/5	
	or	0/5	
4/5	and	3/5	
	or	2/5	
	or	1/5	
	or	0/5	
3/5	and	2/5	
	or	1/5	
	or	0/5	
2/5	and	1/5	
	or	0/5	
1/5	and	0/5	
D.—Experiments in which no result will be obtained.			
0/5	and	0/5	0·0005
5/5	and	5/5	

two doses of a drug, each injected into five animals, one of which would kill 25 per cent. of animals, if a sufficient number were injected, and the other 75 per cent. The table shows that the probability that the LD 75 will kill more than the LD 25 is approximately 0·9215 ; the probability that the same number of animals will die with each dose is 0·0583 ; the probability that more will die with the lower dose is 0·0197, and the probability is 0·0005 that either all will die or all will live with each dose. That is to say, it is only about 11·5 to one that the doses will be placed in their right order, 17 to one they will not be called equal, and about 47 to one that they will not be reversed in order (*i.e.*, on the average, once in 48 tests, the weaker may appear to be the stronger). If 5 animals injected with doses differing by an amount corresponding to these mortalities be used, it is not unlikely that the proper order of the two doses will be reversed in the result. The probability of the reversal is not greater than the limits usually taken to give " significant " differences. If the doses injected are the LD 10 and the LD 90, the chances are 318 to one that the doses will be placed by the test in the proper order, and 609 to one that they will not be reversed in order. For the purpose of standardisation it is clear that the range of error in using 5 animals is almost exactly the difference between the doses necessary to cause 10 and 90 per cent. deaths, and considerably greater than that between the doses causing 25 and 75 per cent. deaths.

Here again, the extent of the error, reckoned as a percentage of the dose injected, depends on the slope and shape of the characteristic. For example, it is possible to confuse doses of cocaine as widely separated as 0·38 mgm. and 0·7 mgm., by using only 5 animals at each dose.

V. *The Construction of a Characteristic Curve.*

The mapping of a characteristic should be done on a batch of animals as far as possible simultaneously. The shape of the curve can be assumed to be the same for groups with different LD 50, as is the case for the summer and winter groups in the diphtheria curves, and the two insulin curves. The part of the characteristic which is of most use for practical purposes is that between the LD 25 and the LD 75. The majority of the animals used should therefore be injected with doses of the drug which will give mortalities between 25 per cent. and 75 per cent. The accuracy of slope of the line drawn through the observed points will be the greatest if the animals are injected in two groups only, one with the LD 25, and the other with the LD 75, as nearly as possible, as will be shown later.

All the calculations given in sections II and III are subject to an error arising from the practical limitations to the number of animals which can be used for setting up the characteristic. It is a matter of considerable difficulty to arrive at an accurate estimation of the error of every part of the curve, but by making a certain number of assumptions a sufficient approximation can be made.

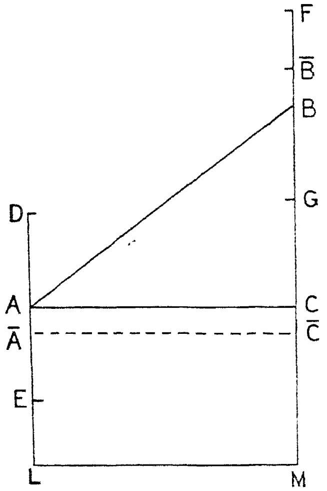

Fig. 9.

Let L and M (fig. 9) represent doses LD 25 and LD 75, and A and B the actually observed values of the percentage mortalities in the two groups injected respectively with these doses. Then A may lie anywhere in the range DE, which represents ± 3 times the s.d. of A on either side of the mean position $\overline{A}$, and similarly B anywhere in the range FG. As on p. 501 the standard deviation of the difference BC of percentage mortality is $100\sqrt{2pq/N}$, where $pq = 0\cdot75 \times 0\cdot25$.

Hence maximum value of BC = true mean value $\overline{BC} + 300\sqrt{2pq/N}$, and minimum value of BC = true mean value $\overline{BC} - 300\sqrt{2pq/N}$.

True value of $\frac{1}{K}$ at L $= \frac{\overline{BC}}{\alpha AC}$, where α is a constant equal to $\frac{100}{\text{dose LD } 25}$.

Therefore AC = 50 $\overline{K}$, where $\overline{K}$ is the true value of K at LD 25. Therefore maximum observed value of

$$\frac{1}{K} = \frac{\text{maximum value of BC}}{\alpha AC} = \frac{1}{\overline{K}} + \frac{300\sqrt{2pq/N}}{50\,\overline{K}} = \frac{1}{\overline{K}} + \frac{6\sqrt{2pq/N}}{\overline{K}}.$$

Similarly minimum observed value of $\frac{1}{K} = \frac{1}{\overline{K}} - \frac{6\sqrt{2pq/N}}{\overline{K}}$.

For insulin, 2,460 mice were used altogether. Taking these for a first approximation as injected in two groups, at the LD 25 and LD 75, and K observed as 1·045, then

$$N = \frac{2460}{2} = 1230, \text{ and the error of } \frac{1}{K} = \pm\frac{6\sqrt{\left(\frac{2\times\frac{1}{4}\times\frac{3}{4}}{1230}\right)}}{1\cdot045} = \pm\,0\cdot102.$$

With K = 1 approximately, the error in K is ± 10 per cent., and since the error of estimation of the dose varies directly with K, this estimated error is probably not more than 110 per cent. of its true value, or less than 90 per cent.

A glance at fig. 9 will show that if doses nearer together than LD 25 and LD 75 are used to determine the slope of the characteristic, the error of K will be increased. If doses further apart than these are used, errors are introduced, owing to the departure of the characteristic from its approximation to a straight line and to the asymmetry of the frequency distribution at the ends of the characteristic.

VI. *Factors which influence the Shape of the Characteristic.*

(*a*) *Multimodal variations.*—In the case in which there is under experiment an animal of which there are two or more varieties, such as male and female with different average lethal doses, but the same range of variability, characteristics with two modes will be obtained. Fig. 10 makes this clearer. Let A and B represent two symmetrical frequency curves, which, we will assume, represent the two varieties of animals. If a series of doses represented along the abscissæ are injected into large enough groups of animals, it is evident that, if the numbers of each variety are equal, a dose of 2·0 units will kill 50 per cent. of the more sensitive animals, and none of the less sensitive, which will correspond to 25 per cent. of the whole number of animals (there being equal numbers of more and less sensitive animals). At a dose of 3 units, there will be roughly

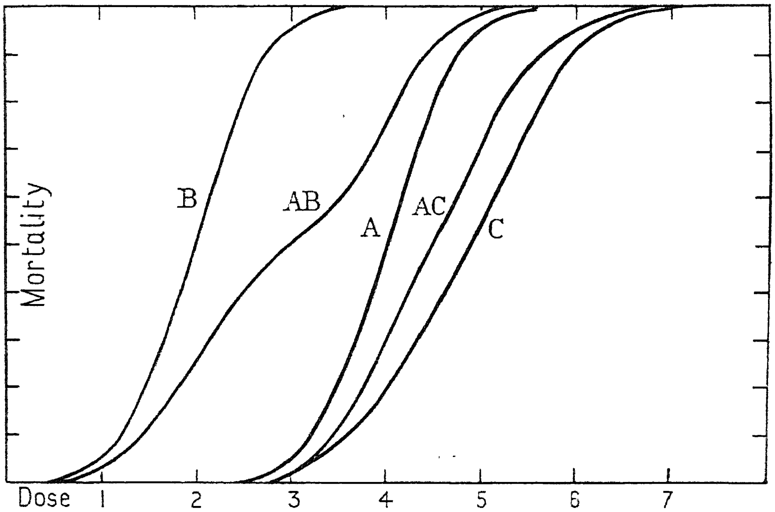

FIG. 10.

96 per cent. dying of the more sensitive, and 4 per cent. of the less sensitive, *i.e.*, 50 per cent. of the total, and so on. In this way, the curve AB is obtained, which has a kink in the middle. Now it is clear that, if only a relatively small number of animals are injected at each dose, for the purpose of determining a characteristic, the observed figures, after allowing for the statistical errors of limited numbers, will apparently fit a smooth characteristic of a slope intermediate between the two, as in the case of diphtheria toxin. Similarly, with A and C, and with combinations A, B, C.

The extreme asymmetry of the dysentery toxin curve is possibly due to the presence of a proportion of individuals which have antitoxin in varying quantities in their serum, the much larger quantities of antitoxin being less frequent in proportion. For dysentery toxin, the sensitivity of the animal to *uncombined* toxin may be taken to be unaffected by the presence of immunity, so that the effect of the antitoxin would be to subtract an equivalent amount of toxin from the dose injected. In consequence, the characteristics for the groups of animals with the same degrees of immunity would be parallel. A series of parallel frequency curves has been drawn in fig. 11, *a*, the one on the extreme left representing the hypothetical characteristic of a non-immune population, and the oblique curve crossing them is the characteristic which would be obtained from a mixture of groups with different antitoxic content, the relative proportion of the various groups being indicated on the base line. So that the extreme asymmetry of the dysentery toxin curve is possibly due to the presence of animals with a different quantity of some neutralising substance in the blood stream. The majority of the animals must have little or none, to account for the steepness of the rise of the curve at low doses. If fewer were non-immune, the characteristic would approach a straight line drawn diagonally across the figure.

(*b*) *Mixtures of toxic effects on the same animal.*—The influence on the form of the characteristic can be predicted under certain conditions. If the drugs act on two separate structures, the susceptibility of which for the poison varies independently, or, if two different poisons acting on separate structures exist in the drug under consideration, the form of the characteristic can be arrived at from the characteristics for each independent effect by the construction given in fig. 11, *b*.

In this figure, A is the characteristic for one toxin, B that for another, and C that for a mixture of the two in equal parts. C is described in the following manner:—At a dose which contains 1 unit of toxin A, y_2 per cent. will die solely as the result of the injection of A, and, of the remainder, y_1 per cent. will

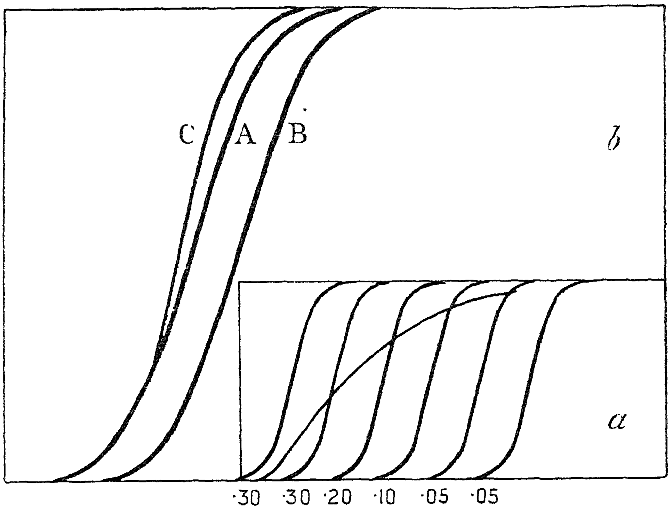

FIG. 11.

die solely as the result of the injection of B, corresponding to $\frac{y_1}{100}(100 - y_2)$ per cent. of the total number injected; therefore, in all, y_3 per cent. $= y_2 + \frac{y_1}{100}(100 - y_2)$ per cent. will die, if the actions of the drugs are completely independent. As a result, the curve C is obtained, which is asymmetrical, in that the "spread" at low dose is greater than that at high. If the action of the two toxins is not independent, *i.e.*, if small doses of B make it easier for A to produce its effect, then the symmetry is not interfered with, but the whole curve is shifted to the left. The asymmetry will be at a maximum when two curves intersect at the LD 50, one being steeper than the other. If the toxins have widely varying median lethal doses, the characteristic obtained would, of course, be identical with the most toxic, if that were present in sufficient quantity.

Variations in the experimental conditions may produce changes similar to those discussed for racial changes. A comparison of the two characteristics for insulin at 37° C. and 28° C. shows that the approximation to a normal curve, produced by the increase in temperature, is very marked (Trevan and Boock, 1926).

It is apparent from these arguments that the more nearly the characteristic, under any given conditions of race, experimental conditions, etc., approaches the normal form, and the steeper it is, the more confidence in its reproducibility for the purposes of test can be obtained; but the best test for reproducibility is, of course, in the end, the experimental test. The number of animals for checking reproducibility need not be as large as that necessary for the setting up of the characteristic.

VII. *Discussion.*

A few points of general interest remain to be cleared up.

(1) The form of a characteristic depends on the combined effect of a large number of different factors, some of which are controllable by alteration of experimental conditions (see, for instance, the remarkable effect of temperature on the response of mice to insulin—Trevan and Boock, 1926) and most of which are not. Among the former is probably the effect of heredity.

It is almost certain that animals inbred, after the fashion of the Wistar Institute rats, will give steeper characteristics than casual groups selected at random from the ordinary dealers' stocks. The effects of colour and weight on the insulin curve have, however, been found to be negligible (Trevan and Boock, 1926), and I do not think very *great* alterations are to be expected by selection of animals. Amongst the uncontrollable factors is the final relationship between dose and effect, on the organs especially affected by the drug, in producing its toxic effect, such as is discussed by Shackell, Gaddum (1926), Clark (1926) and others, using isolated organs. Such dosage-effect relationship must contribute to the complexity of the characteristics obtained, and drugs for which the basal dose-effect curve has a small angle of slope must tend to give characteristics of small angle of slope, and *vice versa.* It is, however, of great importance to realise that the characteristic is not solely a dosage-effect curve in this sense. Its shape depends also, and to a much greater extent, on the variability of the dosage-effect curve itself. Clark, for example, shows that the action of acetyl-choline on muscle may be represented fairly by the formula $Kx = \frac{y}{100 - y}$, where x is dose, and y the percentage of maximum effect produced, and without discussing here whether this agreement entails the acceptance of Clark's explanation of the relation between dose and effect, I may use his figures to explain what I mean. He found that K varied as much as 200 times from one heart to another, and it is this variation of K which chiefly determines the shape of the characteristic, and variations of this order will entirely mask the fundamental dosage-effect curve.

(2) The numbers of animals which are necessary for the determination of toxicity would appear from the above argument to be much larger than it is customary to use. It must be remembered, however, that these errors are worked out for single groups of the dimensions specified. The same number of

animals, split up into smaller groups, and injected with doses along the straight part of the characteristic, will give results which will have only a slightly greater error than that which is obtained by injecting the whole group with the average lethal dose. If, however, any of the smaller groups are injected with doses outside the straight part of the characteristic, the error is considerably increased for these doses. All estimations of toxicity on an unknown sample of a drug must begin by the injection of small groups of animals—two or three animals in each—with a series of varying doses. The doses, for economical working, should be separated by powers of two. Then, at the dose intermediate between those where the break from survival to death comes, a larger group should be put up in comparison with a standard. The size of this group depends on the accuracy with which the final result is required, and that depends on the slope of the characteristic. The number of animals for setting up the characteristic can be reduced by confining the determination of the characteristic to the ends of the straight part, roughly LD 25 and LD 75, and the error allowable in the value of 1/K may in general be as much as 20 per cent. The error of K will then be $+25$ per cent. or $-16 \cdot 6$ per cent., and an error of estimation calculated to be 10 per cent., may be anything between 8·3 per cent. and 12·5 per cent., which is sufficient for most purposes. For insulin, the value of N for setting up a characteristic with an error of K of $\pm$ 20 per cent. is 312, and the total number of animals twice this—624. For digitalis, the total number is 113. Once the characteristic is determined, the number of animals necessary depends on the accuracy desired. One thing should be insisted on, however, with great force, and that is, that unexpected or "discrepant" results must not be eliminated from the final average, except on the clearest evidence of a mistake in technique. If one result is so discrepant that its inclusion or not will make the difference between rejection or acceptance of a sample, the only way to deal with it is to repeat the experiment until the effect of the single discrepancy vanishes in the general average. It is of the utmost importance to observe this rule at all cost.

(3) Finally, with regard to standards in biological assay. For any new method of assay, it must be assumed that the median lethal dose is variable, and that, therefore, comparison must be made with a suitable standard preserved with suitable precautions against deterioration. There may be a minority of drugs which are assayable on the basis of a constant average lethal dose, and, for them, a statement for average lethal dose and the value of K, would be all that was needed for setting up a standard for any given worker, but even when the median lethal dose remains constant, the preparation of a standard is

necessary to correlate the work of different laboratories, and to eliminate the effect of variations in technique and the strain of animals in use.

I am indebted to many colleagues for encouragement and assistance, and amongst these, I would thank particularly Miss Boock, Miss Bainbridge, Dr. O'Brien and Dr. Dale. I also owe a great deal to Dr. Greenwood's advice and criticism.

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Studies on the Relation of Gonadic Structure to Plumage Characterisation in the Domestic Fowl.—III. *The Laying Hen with Cock's Plumage.*

By F. A. E. Crew, Animal Breeding Research Department, University of Edinburgh.

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[Plate 39.]

Among the many fowls exhibiting abnormality of the sex characters which have been presented to this department by breeders sympathetic to the view that a study of the abnormal may lead to a better understanding of the normal processes of development and maintenance, have been two which were in every way similar to the cock which, in 1474, was sentenced by the magistrates of Basle to be burned at the stake "for the heinous and unnatural crime of laying an egg." It is recorded that the executioner on cutting open this cock found three more eggs within him, but this has been doubted by some, who have held that it was impossible and absurd, and that these eggs were not formed by nature, but were created by the fevered imagination of the superstitious. Such was the fate of the sexually abnormal even up to the year 1730. But times have changed, and to-day, instead of being burned at the stake, the offender is called upon to make its contribution to our knowledge of the sex physiology of the fowl.

The bird (No. 2) now to be described, then a year-old Rhode Island Red, was sent to this department because it was thought to be a cock which laid eggs. It certainly at that time had the plumage characteristic of the male of the breed, was laying actively, and had well developed spurs. But it did not crow ; it did not exhibit the male behaviour, but behaved as a female ; normal cocks did not recognise it as a male, nor did the hens ; its head furnishings were typically female in their size (save about the time of the moult, when they shrank until they were as those of a capon) (*see* Plate 39).

Following the moult, the bird assumed the normal plumage characterisation of the hen. In the following year, after the moult, it again became apparelled as a cock. So also in the year after ; in the following year it was again hen-feathered. Throughout these years the bird continued to lay regularly and well. Deliberate plucking of any feather at any time (save immediately preceding the moult in the third and fourth years) was followed by the development of a typically female feather. Post-mortem examination revealed nothing unusual. The bird was a normal hen with an active ovary. The endocrines, in size and structure, were unexceptional.

The explanation of such cases as this would seem to be that these birds are hens ; that at the time of the moult the ovary of the hen undergoes a process of involution, and that for a short period of time the sex-gland is physiologically relatively inactive. If the new plumage begins its growth and differentiation in the absence of the controlling influence of the sex-gland, it will assume the characters of the plumage of a capon. That this is so is seen in the results of experimental gonadectomy. If later, and too late to influence the differentiation of the feathers, the ovary becomes physiologically active again, the plumage as it grows will become tightened (the feathers will not attain the length of those of the capon and will lie much more closely against the body), and the end-result will be a laying hen equipped with a male-type plumage, since the plumage of the agonadic bird of either sex is of this type. It is possible that it is not the ovary that is solely responsible, for thyroidectomy of the hen-feathered cock is followed by the development of male-type plumage. It is not improbable, therefore, that a temporary functional insufficiency on the part of the thyroid is responsible for the development of male-type plumage in these cases.

The bird which has male plumage and which lays eggs is not a cock at all, then, but a hen, normal in every way, save that her plumage is as that of a capon, tightened by the physiological action of certain of the endocrine glands. The disharmony in plumage characterisation is due to a temporary disfunctioning of the ovary (and/or the thyroid) at the time of the moult. That this is so is shown by the facts that plucked feathers are replaced by typically female feathers, and that the same bird, following moults in different years, does not always assume male plumage.

It is unfortunate that the birds of this kind so far examined have belonged to self-coloured varieties. Had they been parti-coloured, Brown Leghorn for example, the study would have been much more fruitful, for then the transformation in the individual feather from one sex type of structure and coloration to the other could have been followed.

The observations of Punnett and Bailey (1921) regarding inheritance of hen-feathering in cocks bear directly upon the question now being considered. Among the offspring of the mating of cock-feathered and hen-feathered breeds they obtained many males which exhibited an intermediate type of feathering. These intermediates, of many grades, became fully henny, or nearly so, following the first moult; and some of them at subsequent moults reverted to the intermediate type. These facts can be brought into line with the suggested interpretation offered in the case of the cock-feathered laying hen, if it is assumed that these intermediate cocks were genetically hen-feathered and that the expression of the factors for hen-feathering was conditioned by the fact that, at the time of the assumption of plumage, the mechanism (gonad-thyroid) which controls plumage growth and differentiation is not stabilised, or temporarily becomes disturbed.

Nonidez (1924), in discussing Morgan's (1919) suggestion that hen-feathering in cocks was due to the action of an internal secretion elaborated by the "luteal" cells of the gonad, enunciates the view that it is due not to the physiological influence of the gonad but to that of some endocrine gland, *e.g.*, thyroid and pituitary.

Buchanan (1926) has described a Dorking cock which, when two years old, assumed hen-feathering, the change being practically complete after a single moult. Whilst under observation he never was seen to copulate and all the eggs from his pen were infertile. Post-mortem examination revealed abnormal testes and thyroid, the condition of the latter suggesting deficient functioning. She also examined the thyroids from normally hen-feathered Sebright bantam cocks and states that the histological structure of these glands was such as to suggest deficient functioning. From a consideration of these facts, she puts forward the suggestion that, in the fowl, the physiological actions of thyroid and gonad are mutually antagonistic, deficiency of that of the gonad leading to maleness, deficiency of thyroid leading to femaleness.

The work of Torrey and Horning (1925), of Zavadovsky (1925), of Cole and Reid (1924), and of Brambell (1926) bears directly upon this question of the interrelation of gonad and thyroid in respect of plumage characterisation, and though as yet the exact nature of this interrelationship is not known, it has become necessary, in any discussion of plumage characterisation, that thyroid influence as well as gonadic be considered.

The fact that the bird described above had well-developed spurs may not be without significance. Spurs are not usually developed by the hen. In certain strains, however, the hens commonly have spurs; this character has a genetic basis, and in such cases the hens are unexceptional layers. It is possible that this hen genetically was so constituted that she would have developed spurs in the absence of any abnormality of ovarian or thyroid functioning. Spurs are usually developed by those hens which suffer from pathological conditions of the ovary, and it would seem that any hen will develop spurs under certain conditions. It is possible that the physiological activity of the ovary is the inhibitor of spur-growth; that a prolongation of the phase of relative physiological inactivity of the ovary at the time of the moult allows spur-growth to commence; and that when once begun, growth will continue in response to the stimulus of nutrition and will not be completely checked by the revived activity of the ovary.

Goodale (1922) was able to obtain, by selection, a strain of fowls the females of which were spurred. Crosses between this strain and a breed in which the hens were never spurred gave a spurless F_1. Segregation occurred in F_2, the ratios indicating more than a single factor difference.

In this connection, it is interesting to note that Pézard (1915) found that ovariotomy results in the development of spurs, as in the male, contrary to the observations of others. Pézard records that non-ovariotomised hens, which occasionally develop spurs, are usually poor layers. He agrees, however, that the development of spurs is to be attributed to an insufficiency, or a temporary cessation, of ovarian function, as indicated by scarcity of eggs laid. He regards the absence of spurs as being due to an inhibitory action of the ovary, and his conclusion is supported by the fact that spur-growth in castrates is inhibited by ovarian grafting.

It is of interest to note that abnormality in feathering, taking the form of cock feathering in the hen, may be sufficient to make the individual sexually unattractive to cocks, and that, as a consequence of this, the eggs which it lays are unfertilised.

The second bird (No. 1), also a Rhode Island Red, which is still under observation, has presented an exactly similar history. Two years ago she came to the Department, a laying hen with cock's plumage. Since then, with the years, her plumage has been in its characterisation female, male, and now is again female.

Summary.

The cock-feathered laying hen is a female, normal in every respect save that her plumage becomes as that of the agonadic bird following the moult, as the result of a transient disfunctioning of the ovary (and/or of the thyroid) at this time.

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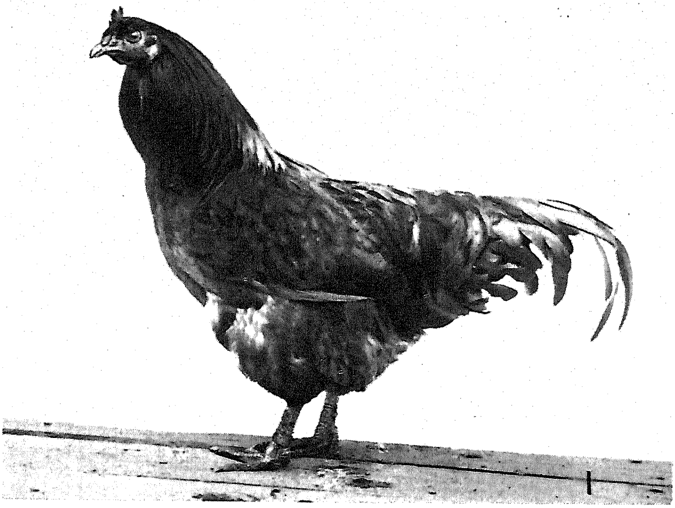
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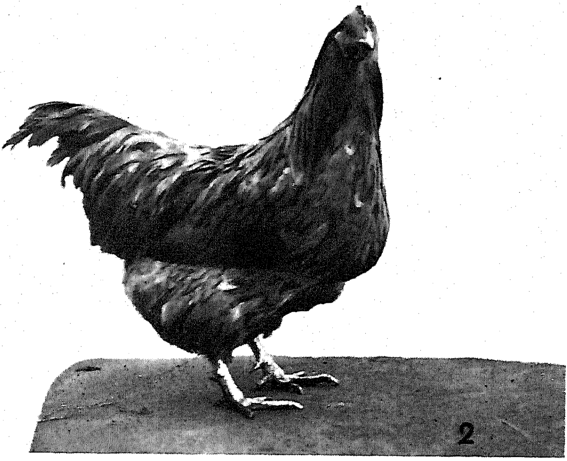
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The Isolation of Some Hitherto Undescribed Products of Hydrolysis of Proteins.—Part IV.

By S. B. Schryver, and H. W. Buston, Department of Biochemistry, Imperial College of Science and Technology, London.

(Communicated by Prof. V. H. Blackman, F.R.S.—Received April 29, 1927.)

In the course of certain researches on the chemistry of gelatin carried out in this laboratory, it was found* that the Hausmann numbers obtained for a given sample of gelatin showed considerable variations, depending upon the preliminary treatment of the sample. In particular, the diamino-nitrogen (*i.e.*, the nitrogen precipitated by phosphotungstic acid) was found to vary from about 20 per cent. to as much as 30 per cent. of the total nitrogen. It was found that if the gelatin were hydrolysed directly with boiling acid, a constant value for the diamino-nitrogen, of the order of approximately 20 per cent. of the total nitrogen, was obtained; if, however, the gelatin were allowed to stand with acid in the cold, prior to hydrolysis, the value for the diamino-nitrogen rose considerably, in some cases by as much as 9 per cent. It has therefore been the practice in this laboratory, in hydrolysing a protein, to add the protein to the boiling acid, thus minimising any effect produced by standing with acid prior to hydrolysis; by this method, consistent results are obtained. Further, it was suggested by Knaggs (*loc. cit.*) that the increased nitrogen found in the diamino-fraction was due to the formation of peptides, which were resistant to hydrolysis, and were precipitated by phosphotungstic acid.

The present research had for its object the investigation, qualitatively and quantitatively, of the substances (peptides or other) causing this increase in diamino-nitrogen. In the course of the work, no peptides were isolated, and no indication of their presence was discovered; the whole of the increased diamino-nitrogen could be accounted for by (*a*), increases in two of the bases (lysine and arginine) known to be present, and (*b*) the presence of a base (*dl*-lysine) which has not previously been recognised as a normal hydrolysis product of the proteins, but which has been prepared by other means. The properties of this base are therefore well known. It does not apparently arise by mere racemisation of the lysine already known to be present in the protein, as ordinary active lysine was also obtained, in quantity rather more than normal. The origin of this base is still unknown. It is probably derived from some other unknown constituent of the protein, from which it is set free by the action of acids acting only in the cold.

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* Knaggs, 'Biochem. Jl.,' vol. 17, p. 488 (1923).

Experimental.

Preliminary experiments.—Preliminary experiments were made on a small scale, using about 8–10 grams of Coignet's Gold Label gelatin in each case. Four samples were treated as follows:—

(1) Boiled immediately with 25 per cent. sulphuric acid for 20 hours.

(2) Allowed to stand for 24 hours with 25 per cent. sulphuric acid in the cold; then boiled for 20 hours.

(3) Allowed to stand for 24 hours with 25 per cent. sulphuric acid at 0° C.; then boiled for 20 hours.

(4) Allowed to stand for 24 hours with water in the cold; then boiled with 25 per cent. sulphuric acid for 20 hours.

In each case, the diamino-nitrogen was precipitated after hydrolysis by means of phosphotungstic acid in 5 per cent. sulphuric acid solution; the results obtained are given in Table I below.

Table I.

——	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 4.
Total nitrogen in hydrolysis liquid	1·254 g.	1·084 g.	1·080 g.	1·108 g.
Nitrogen in filtrate from phosphotungstate ppt.	0·992 g.	0·766 g.	0·764 g.	0·883 g.
Hence nitrogen pptd. in diamino-fraction	0·262 g.	0·318 g.	0·316 g.	0·225 g.
Percentage of total nitrogen in diamino-fraction	20·90	29·36	29·32	20·34

The bases, after liberation from their phosphotungstates, were further separated (in experiments 1 and 2 above) by the usual method of Kossel, using silver sulphate and barium hydroxide, the nitrogen in each of the three fractions thus obtained being estimated. Table II shows the distribution of nitrogen among the so-called "histidine," "arginine" and "lysine" fractions.

From these figures it would seem that the main increase in the diamino-nitrogen is due to the presence of some substance giving a silver salt insoluble in alkaline solution, resembling arginine in this respect, a much smaller increase being due to a substance giving a soluble silver salt (lysine fraction). It will be noted that there is no increase at all in the histidine fraction.

Table II.

—	Percentage of total nitrogen or protein :—	
	Expt. 1 (hydrolysed immediately).	Expt. 2 (treated with acid before hydrolysis).
	Per cent.	Per cent.
Histidine-fraction	1·63	1·59
Arginine-fraction	11·83	15·97
Lysine-fraction	6·65	7·95

Experiments were now carried out on a much larger scale with the object of determining qualitatively the nature of the substances causing these increases ; it was found inconvenient to make these experiments of a quantitative nature. A further series of experiments was performed later to place the work on a quantitative basis.

Gelatin (500 grams) was allowed to stand overnight with 25 per cent. sulphuric acid in the cold ; the liquid was then boiled for 20 hours to hydrolyse the protein, after which it was rapidly cooled, diluted with water to such an extent that it contained 5 per cent. of sulphuric acid, and the basic portion was then precipitated with phosphotungstic acid reagent. The precipitate was allowed to stand overnight, filtered off, washed, and decomposed by barium hydroxide. The solution of the bases so obtained was fractionated by means of silver sulphate and barium hydroxide in the ordinary manner, and the so-called histidine, arginine and lysine fractions were freed from silver, barium, etc. The histidine and lysine fractions were not in this case investigated further, attention being concentrated on the arginine fraction, which shows the main increase in quantity.

Investigation of the " arginine fraction."

A method of precipitating arginine quantitatively has recently been introduced by Kossel and his collaborators,* based on the fact that arginine gives a salt with flavic acid (1-naphthol, 2·4-dinitro, 7-sulphonic acid) which is completely insoluble in cold 5 per cent. sulphuric acid. Accordingly, the arginine fraction was precipitated with this reagent (5 per cent. aqueous flavic acid) in the presence of sulphuric acid. After vigorous shaking, precipitation commences, and a heavy precipitate of orange-yellow crystals rapidly forms. After standing for 24 hours, the crystals were filtered off and washed with weak flavic acid in sulphuric acid. The combined filtrates and washings were treated with barium hydroxide to remove most of the flavic acid, and the base in the filtrate was precipitated with phosphotungstic acid in the usual manner, and liberated by barium hydroxide. The solution of the free base was evaporated to a fairly small bulk (containing about 1 per cent. of nitrogen), and was found readily to yield a crystalline picrate, sparingly soluble in cold water. The whole of the solution was therefore treated with hot aqueous picric acid, warmed on the steam-bath, and left to crystallise. The picrate separated in clumps of golden-brown fine needles, and was washed with a little ether and recrystallised from water. (In some cases the picrates separated first in the form of an oil, which however rapidly became crystalline on standing). The crystals were of a very different form from those of arginine picrate, which was prepared for comparison. The latter formed very long and thin silky needles, of a lighter colour than those of this unknown base.

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* Kossel and Staudt, ' Zeit. Physiol. Chem.,' vol. 156, p. 270 (1926).

The free base was obtained from the picrate by the usual method, and was dried at 100° *in vacuo* over phosphorus pentoxide and solid caustic potash. Combustion of the base and determination of its nitrogen by Kjeldahl gave results that were inconsistent with any formula, but which were near those required by lysine ($C_6H_{14}O_2N_2$). From the figures obtained it seemed possible that the substance might be lysine, contaminated with a small amount of histidine. The well-known colour reaction for histidine, with diazobenzene-sulphonic acid, was strongly positive. On treating a solution of the base with mercuric chloride, a small precipitate was obtained, and was removed. The filtrate, after being freed from mercury, no longer gave the colour reaction for histidine. The base present in the filtrate was again converted into the picrate.

The picrate thus obtained, after recrystallisation, melted at 225° C., with decomposition. 1 part was soluble in about 200 parts of water at ordinary temperature, but the salt was readily soluble in hot water. An estimation of the equivalent of the base was made by the nitron method, the following results being obtained :—

(1) 0·0667 g. of picrate gave 0·0965 g. of nitron picrate.

(2) 0·5986 g. of picrate gave 0·8701 g. of nitron picrate.

Hence equivalent of base = (1) 145·0 ; (2) 143·5. Average, 144·25.

The base was liberated from the picrate in the usual manner, the solution of the free base was evaporated to a syrup, and granulated with absolute alcohol ;

the solid was dried at 100° *in vacuo* over phosphorus pentoxide and solid caustic potash. Combustion of the free base to determine the carbon and hydrogen present gave :—

(1) 0·003156 g. gave 0·002667 g. water and 0·005665 g. carbon dioxide.
(2) 0·003424 g. gave 0·002912 g. water and 0·006155 g. carbon dioxide.

Found :—C = 48·96 per cent., 49·03 per cent. ; H = 9·39 per cent., 9·45 per cent. ; N = 19·29 per cent., 19·11 per cent. (Kjeldahl).

The above figures agree with the formula $C_6H_{14}O_2N_2$; this requires :—

C = 49·27 per cent. ; H = 9·60 per cent. ; N = 19·18 per cent.

The equivalent required by this formula is 145. The formula given above is that of lysine, but the base here obtained differs from ordinary lysine in being precipitated by silver sulphate and barium hydroxide.

The benzoyl and phenyl hydantoin derivatives were prepared. these being readily obtained and well-defined crystalline bodies. In the case of the former, the method given by Lawrow* for the preparation of dibenzoyl lysine was used. 1 g. of the base was dissolved in 100 c.c. of 10 per cent. caustic soda solution, and 12 c.c. of benzoyl chloride added. The liquid was kept cool and vigorously shaken, till the smell of benzoyl chloride could no longer be detected. 500 c.c. of light petroleum were then added, and 10 per cent. hydrochloric acid was run in till no further precipitate was produced. The whole was thoroughly shaken, and the solid which separated was filtered off. It was washed once with petroleum, then with warm water and recrystallised from a large amount of hot water ; the white crystals were dried over sulphuric acid *in vacuo*. The crystals were soluble in alcohol, moderately soluble in ether, and insoluble in cold water. Their melting point was 145° C. Combustion gave the following figures :—

(1) 0·003153 g. gave 0·001778 g. water and 0·007867 g. carbon dioxide.
(2) 0·003488 g. gave 0·178 c.c. nitrogen at 14° C. and 755 mm.

Found :—C = 68·04 per cent. ; H = 6·23 per cent. ; N = 7·88 per cent.

The formula $C_{20}H_{22}O_4N_2$, representing a dibenzoyl derivative of a base with the formula $C_6H_{14}O_2N_2$, requires :—

C = 67·80 per cent. ; H = 6·21 per cent. ; N = 7·91 per cent.

The phenyl hydantoin of the base was prepared by the method given by Herzog for the preparation of lysine phenyl hydantoin.† 1 g. of the base was shaken with 12 c.c. of N. caustic soda and 2 g. of phenyl isocyanate. The mixture was left for 4 hours, and precipitated with hydrochloric acid. The white precipitate was evaporated in small amounts with 10 c.c. of concentrated hydrochloric acid on the steam-bath. The residue was recrystallised from a mixture of alcohol and acetone (7 : 1), when the phenyl hydantoin was obtained in the form of a bulky mass of white needles. The recrystallised product was found to have a m.p. of 192° C. Combustion of the product gave the following results :—

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* Lawrow, 'Zeit Physiol. Chem.,' vol. 28, p. 585 (1899).

† Herzog, 'Zeit Physiol. Chem.,' vol. 34, p. 525 (1902).

(1) 0·002223 g. gave 0·001225 g. water and 0·005353 g. carbon dioxide.
(2) 0·003006 g. gave 0·001647 g. water and 0·007234 g. carbon dioxide.
(3) 0·003120 g. gave 0·403 c.c. nitrogen at 12° C. and 759 mm.

Found :—C — 65·70 per cent., 65·64 per cent.; H — 6·13 per cent., 6·08 per cent.; N — 15·24 per cent.

The formula of the phenyl hydantoin of a base $C_6H_{14}O_2N_2$ would be $C_{20}H_{22}O_3N_4$, and would require :—

C — 65·57 per cent.; H — 6·01 per cent.; N — 15·30 per cent.

The constitution of the free base, as well as the above analyses of derivatives and of the picrate, would indicate that the base is lysine. The melting point of the phenyl hydantoin is however some 10° higher than that of the corresponding derivative of lysine, but agrees with that of the phenyl hydantoin of *dl*-lysine (Herzog). The free base was tested in aqueous solution for its optical activity and found to be entirely inactive. It would seem therefore that the base causing the increase in the "arginine-fraction" is actually *dl*-lysine; this substance is known to give a silver compound insoluble in alkaline solutions.

The flavic acid precipitate.

In order to make certain that the precipitate produced by flavic acid contained no other bases besides arginine, it was decomposed by repeatedly extracting its hot (60°–70°) solution in 5 per cent. sulphuric acid with butyl alcohol, having first recrystallised the flavate from hot sulphuric acid of this strength. (It has been shown by Pratt* that only pure arginine flavate recrystallises from acid of this concentration.) The mother liquor remaining after recrystallisation of the flavate was decomposed separately, and found to contain a small amount of a base, which was identified as *dl*-lysine. The quantity of this base amounted to rather less than 5 per cent. of the total bases present in the flavate precipitate. The free base from the recrystallised flavate agreed in properties, nitrogen-content, etc., with arginine; no other base was found.

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* Pratt, 'J. Biol. Chem.,' vol. 67, p. 351 (1926).

The lysine-fraction.

The lysine-fraction (*i.e.*, that portion of the bases not precipitated by silver sulphate and barium hydroxide) was investigated, and found to yield only active lysine. This was isolated in the usual way by means of the picrate, which was recrystallised from hot water, and melted at 220° C. The optical activity of the carbonate in aqueous solution was determined, and found to agree with that of active lysine. The phenyl hydantoin of the base, prepared as above, melted at 184–5° C., agreeing with the phenyl hydantoin of active lysine.

Quantitative experiments.

Experiments were carried out to determine the nitrogen distribution among the various bases found in the diamino-fraction, the previous work having been confined to qualitative experiments for reasons of convenience.

Two portions each of 50 g. of Coignet's Gold-Label gelatin were taken; one was hydrolysed immediately with 25 per cent. sulphuric acid for 20 hours; the other sample was soaked for 24 hours with the cold acid and then similarly hydrolysed. The bases were obtained from the hydrolysis mixture in the usual way. In each case, a portion of the bases (about one-sixth) was treated by the silver sulphate—barium hydroxide method, to determine the distribution of nitrogen. The results are tabulated below:—

Table III.

—	Expt. 1 (hydrolysed immediately).	Expt. 2 (treated with acid before hydrolysis).
Total N in hydrolysis liquid	7·02 g.	7·28 g.
N in basic ppt.	1·32 g.	1·94 g.
Percentage of total nitrogen in basic fract.	18·55 per cent.	26·65 per cent.
N pptd. in histidine-fraction*	1·70 ,,	1·73 ,,
N pptd. in arginine-fraction*	10·59 ,,	16·71 ,,
N in lysine-fraction*	5·21 ,,	6·86 ,,

* Calculated as percentage of total nitrogen in protein.

The remainder of each basic-fraction was treated similarly in each case, the procedure being as follows:—Arginine was first precipitated by flavic acid, the precipitate being recrystallised from hot 5 per cent. sulphuric acid; the filtrate, freed from flavic acid, was precipitated with mercuric chloride to remove histidine, and then with silver sulphate and barium hydroxide to separate any residual arginine, and *dl*-lysine, from active lysine. The silver precipitate was decomposed, and the solution of the free bases was again precipitated, after concentration, with flavic acid, a small amount of precipitate being given in each case. After removal of the excess of flavic acid, the nitrogen was determined in the liquid, the value obtained being taken to represent the amount of *dl*-lysine present. The results are given in Table IV.

Table IV.

—	Expt. 1.	Expt. 2.
	Per cent.	Per cent.
N in arginine-fraction*	10·59	16·71
N in above fract. not pptd. by flavic acid (*i.e.*, *dl*-lysine)	0·95	5·10
Hence actual arginine	9·64	11·61
N in lysine-fraction	5·21	6·86
N actually recovered as lysine ...	4·98	5·54

* As given by small scale experiment, Table III.

(All values are expressed as percentage of total nitrogen of protein.)

It will be seen from the above that a small amount of nitrogen remains unprecipitated by flavic acid from the arginine fraction of the sample hydrolysed immediately (Expt. 1), even though the precipitation was carried out twice. This is considered to be mainly *dl*-lysine, though a little arginine is still probably present. In any case, the amount of *dl*-lysine present in the unsoaked gelatin does not account for more than 0·95 per cent. of the total nitrogen present. A small amount of *dl*-lysine would naturally be expected.

The above results (Tables III and IV) are summarised in the following table:—

Table V.

—	Gelatin hydrolysed immediately.	Gelatin treated with acid.	Increase.
	Per cent.	Per cent.	Per cent.
Total diamino-N	18·55	26·65	8·10
Histidine	1·70	1·73	0·03
Arginine	9·64	11·61	1·97
Lysine	5·21	6·86	1·65
dl-lysine	0·95	5·10	4·15

Total increases accounted for, 7·80 per cent.; unaccounted for, 0·30 per cent.

(All values expressed as percentages of total nitrogen in protein.)

Summary.

(1) Till recently it was considered that the basic-fraction obtained from the hydrolysis products of proteins contained only histidine, arginine and lysine. It has now been shown that three other bases may be present, viz., hydroxylysine,* protoctine,† and *dl*-lysine.

(2) The *dl*-lysine does not arise from racemisation of the active lysine normally present, but is probably derived from some unknown constituent of the protein, from which it is only liberated by the action of acids acting in the cold.

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* 'Proc. Roy. Soc.,' B, vol. 98, p. 58 (1925).

† 'Proc. Roy. Soc.,' B, vol. 100, p. 360 (1926).

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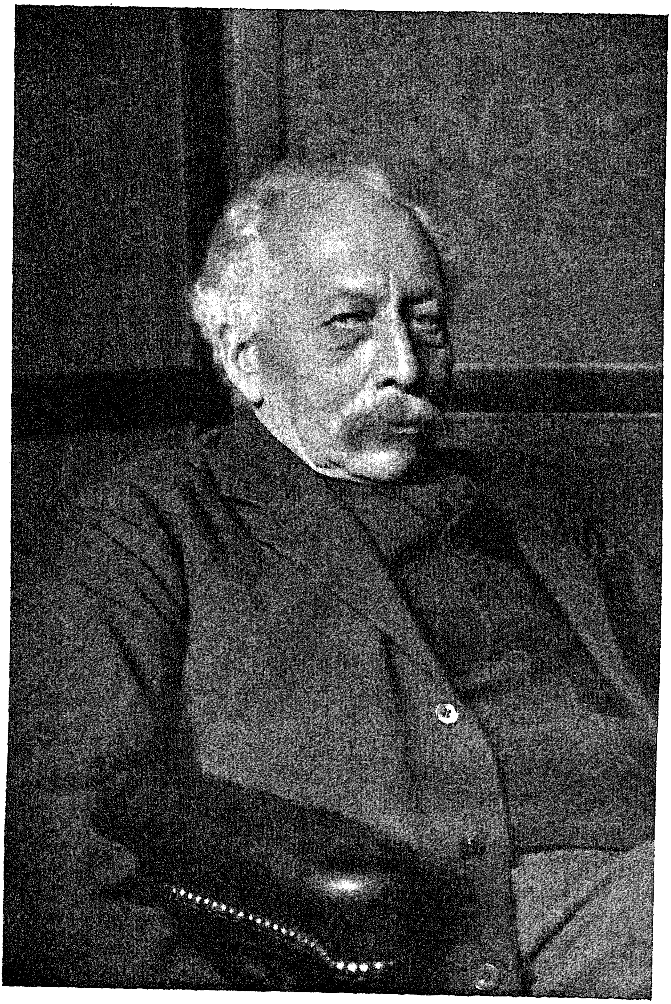


W. Bateson

WILLIAM BATESON—1861-1926.

WILLIAM BATESON died on February 8th, 1926, and English science lost in him one of her really great men. He was born at Whitby in 1861, the son of the Rev. W. H. Bateson, D.D., Master of St. John's College, Cambridge. He was educated at Rugby and St. John's College, and was attached to the study of Zoology. He was elected Balfour Student in 1887, and his early research work on Balanoglossus marked him as one of the coming men in biological science. His early University life coincided with the general settling down of educated opinion in favour of the Darwinian proofs of the validity of evolution as affording the key to the mystery of the origin of species. But with the general acceptance of that doctrine much was taken for granted, and all the foundations for the new belief were apt to be accepted as sound beyond the need of further examination.

Bateson, however, was one of the few who were not content with the flow of current opinion, nor with the methods of investigation which were being pursued, more especially in this country. He had the, to many minds, disconcerting gift of facing and concentrating on the difficulties that were too often slurred over by his contemporaries, and he set himself to probe into the fundamental assumptions that lay at the root of the theory of evolution as then understood. Chief amongst these fundamental assumptions was the notion that the basis of evolutionary change in living forms lay in the gradual summation of almost imperceptibly small variations, and that, in fact, specific change was attributable to selection and accumulation of these small variations as the result of environmental conditions.

Bateson's investigations (in the course of which he was, incidentally, led to visit Turkestan) led him to formulate a very different possibility, based on direct observation and crystallised in his term of "Discontinuous Variation." The wide range of observations, and the conclusions he drew from them, are embodied in a considerable volume, modestly entitled "Materials for the Study of Variation," published in 1894. The book was before its time. It aroused intense hostility in the minds of some, and lay neglected by others who perhaps were unprepared to receive the new points of view, or at any rate were too much enthralled in the chains of the new evolutionary orthodoxy to appreciate the immense importance of Bateson's real meaning.

The book was really a wonderful production, and it was not its author's fault that its great importance was not grasped at once. The whole position was set forth with a lucidity and force that ought at once to have arrested general attention, but the rigidly scientific presentation ran counter to the facile teleology which, like a noxious weed, had overgrown the solid framework of evolutionary doctrine. Bateson himself rightly urged that his own views (based as they were on the new and critically presented evidence which he himself had done so much to render available) in no way detracted from the great and epoch-making work of Charles Darwin, but he found himself often in conflict with his earlier contemporaries. The reason was mainly that he ever sought to submit theory and hypothesis to the touchstone of fact: "the readiest way, then, of solving the Problem of Evolution is to study the facts of Variation." In his "concluding reflexions" (chapter XXV) he summed up the matter by saying ". . . . a presumption is created that the discontinuity of which species is an expression has its origin not in the environment, nor in any phenomenon of adaptation, but in the intrinsic nature of organisms themselves, manifested in the original Discontinuity of Variation" (p. 567). How far he was in advance of the opinions current at that time will be readily understood by those who have witnessed the slowness with which the old teleological attitude of mind has been ousted from circles into which it ought never to have gained entrance at all. On a later page occurs the pregnant passage—"The only way in which we may hope to get at the truth is by the organisation of systematic experiments in breeding . . . sooner or later such investigation will be undertaken, and then we shall begin to know."

The passage just quoted was written more than six years before the disinterment, in 1900, of Mendel's researches from the wholly undeserved oblivion into which they had fallen.

It is right to refer to this earlier work at some length (though indeed much more could well be said about it) since it indicates the key to that fascination which he exercised upon a growing number of young workers. He was an inspiring man, earnest in his seeking for truth, impatient of sloppy work or of hasty conclusions, always insistent on accuracy of observation and on the proving of each step in an investigation.

When sufficiently roused he was capable of a somewhat fierce polemic, as is shown in his pamphlet entitled "Variation and Differentiation" privately printed in 1902, which was directed against his adversaries amongst the biometricians. The force with which he sustained his thesis in this and other writings probably did more to put the biometric school of thought into its right position in evolutionary enquiry than any other contributions to the literature of the subject.

As soon as the researches (1865–69) of Mendel were brought to light in 1900, Bateson was obviously already in a position to grasp their immense significance for the experimental study of evolution. In 1905 he visited Brün, the scene of Mendel's investigations, in the hope of lighting on other papers or notes which were known to have been in existence, but unfortunately the search was fruitless.

Bateson threw himself into the new work with all his force, and, characteristically wide in his range of enquiry, greatly extended the scope of his investigations on plants. Perhaps it is in no small degree owing to his contact with zoological *together with botanical* evidence, that he was enabled to achieve so much and to preserve so fine a balance of mind. His book on "Problems of Genetics," published in 1913, stands out as a landmark of the first importance, and it will always appeal to the student of genetics whether he be mainly a zoologist or a botanist.

Between the years 1902 and 1909 five valuable reports (in addition to the publication of other memoirs) were made to the Evolution Committee of the Royal Society. The first four deal with work carried on mainly in association with Miss Saunders and Mr. (now Professor) Punnett. Meanwhile his fame had spread to other lands, and he visited America as Silliman Lecturer in 1907. In 1908 he was appointed Professor of Biology at Cambridge, a post which he held until the end of the following year, when he accepted an invitation to become the first Director of the John Innes Institution at Merton.

It is difficult to overestimate the importance of this decision, for although it necessarily entailed attention to administrative work, during the sixteen years in which he directed its activities, he made the Institution one of the most famous centres for the study of genetics in the world. This is perhaps hardly the place to indicate what he accomplished in carrying out the intentions of Mr. John Innes, who by his will founded and endowed the Institution for the advancement of Horticulture. Suffice it to say that during Bateson's directorate the place has been enlarged more than once, greenhouses have been built for the cultivation of plants and for the scientific prosecution of genetic studies, while a fine laboratory and library afford ample facilities for a number of workers who, attracted by Bateson's reputation and output of scientific work, have come from many countries to carry out research there. Nor did he lose sight of the other aspects of the objects of the Institution which, *inter alia*, include the training of practical horticulturists. It may truly be said that starting from the very beginning, owing to Bateson's stimulating genius, his wide range of biological interests, and his scientific foresight, the work of the Institution has not been limited to purely academic studies but has extended so as to include scientific horticultural and agricultural subjects, and hence to assist commercial interests as well. The high hopes that were entertained as soon as it became known that the Institution was placed under the charge of the foremost experimental biologist of his time, have been thus more than justified.

Amongst the many lines of work which have been initiated and encouraged by him, the important series of investigations on Somatic Segregation demands special mention, for he has shown that there is evidence calling for pause in accepting, as unreservedly and universally true, the view that the factors responsible for the characters of individual offspring are invariably and solely segregated at meiosis. His work, in collaboration with Miss Pellew, upon the roguing of certain races of peas is disturbing to the peace of those whose minds seek for uniformity, and although the riddle itself still remains unsolved there can be no doubt but that its ultimate solution is of the highest scientific importance.

Bateson in earlier days had recognised the linkage of characters, a phenomenon which is now widely recognised, and the study of which has yielded such rich results in connection with the cytological researches on Drosophila, at the hands of Morgan and his co-workers at Columbia University. Bateson himself always maintained an attitude of some reserve towards the results of cytological investigations in relation to their interpretation in terms of genetics. Whilst freely recognising the cogency of much of the evidence drawn from the study of cell and nuclear division, in his last years he seemed again to adopt a more cautious attitude towards it. He expressed very plainly this feeling of reserve in the last paper published by him before his death (" Segregation," 'Journal of Genetics,' 1926).

Other lines of work carried out by him at Merton dealt with the genetics of Primula, the breeding of a tall race of flax for longer fibres, and of sugar-beet and mangels which should be free from the bad habit of " bolting," *i.e.*, flowering prematurely. In both of these last-named investigations he was eminently successful, and the work was at once recognised as fraught with great and immediate commercial possibilities.

As the foregoing lines may have indicated, Bateson was always in full sympathy with the *practical* application of genetics in all its various branches, and hence it was only natural that he should have been a welcome visitor wherever practical genetics formed an essential part of the business of individuals or firms engaged in the various operations of breeding and selection, whether of animals or plants. In this way he did much to cement good relations between science and industry in several directions, but perhaps in none more strikingly than in connection with the great horticultural establishments. One may be permitted to hope that the co-operation, mutual interest and confidence, which he did so much to promote, may continue to flourish to the common advantage both of science and practice.

In his later years Bateson devoted much study to the phenomenon of Variegation, and several important papers on this subject have appeared from the John Innes Laboratory in the 'Journal of Genetics,' which was founded by him in 1910.

It is not surprising that Bateson's eminence as a biologist of the first rank should have met with wide recognition. He was elected an Honorary Fellow of his old College (St. John's) at Cambridge, he became a Fellow of the Royal Society in 1894 and served on the Council 1901–03. He was awarded the Darwin Medal in 1904, and a Royal Medal in 1920. In 1904 he was President of Section D of the British Association, and President of the Association itself in 1914 when it met in Australia, and the stimulating Presidential Addresses delivered at Melbourne and at Sydney were in keeping with the highest traditions of the Association. He was appointed Fullerian Professor of Physiology in the Royal Institution 1912–14, and he delivered the Croonian Lecture to the Royal Society in 1920, taking "Gametic Segregation" as his subject. In 1922 he delivered the Joseph Leidy Memorial Lecture in America, again dealing with the problem of segregation.

He was a Trustee of the British Museum, and in this connection reference may be made to another side of his many intellectual activities. He was keenly interested in art, and was a recognised authority on the work of Oriental artists. He himself possessed a collection of high quality, and after his death his wife, Mrs. Bateson, who survives him, presented to the British Museum a number of picked specimens which will be placed in the Department of Ceramics and Oriental Antiquities, as well as others which will be found in the Department of Prints and Drawings. This generous gift has been recognised by the Museum as consisting of things it "could hardly have hoped to acquire by purchase."

Bateson possessed in a high degree the quality, easier to perceive than define, which is sometimes called "distinction," but perhaps is better expressed by *ἀρετή*. It was impossible to hold converse with him without becoming aware of his unconscious possession of this quality. He was a source of inspiration to all who were privileged to work under his guidance, and his own past achievements in science will ever remain as an enduring monument to his memory.

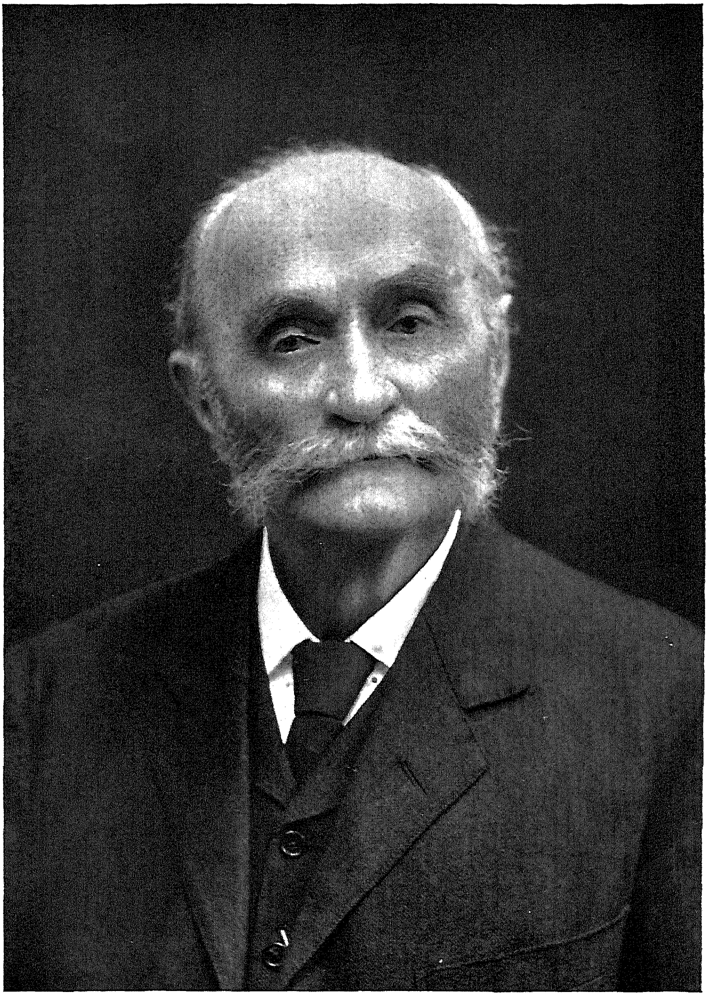
J. B. F.

SIR WILLIAM SCHLICH, K.C.I.E.—1840-1925.

Wilhelm Schlich, son of Daniel Schlich (1792–1875) and his wife Charlotte Frank (1801–67), was born at Flonheim in Rheinhessen, where his father was Lutheran pastor, on February 28, 1840; both parents belonged to Hessian families. The seventh child in a family of nine, Wilhelm had not yet gone to school when the household migrated from Flonheim to Langgoens, near Giessen, in the year of troubles, 1848. A man of many gifts, as to German and French *sermonis utriusque linguae doctus*, Pastor Schlich added to a scholarly knowledge of Latin, Greek and Hebrew, an informed interest in agriculture and reasoned views regarding education. At the jubilee of his pastorate in 1865, the Grand Duke of Hesse-Darmstadt appointed him Kirchenrat.

Schlich's elementary teaching was undertaken largely by his father and was continued until he was eleven. Preparatory education began in 1851 at a school near Darmstadt from which he was transferred later to another at Essenheim in Rheinhessen. In 1854 he was sent to a third school at Schotten in Oberhessen, where he studied until autumn, 1855, when he entered the Darmstadt Technical High School. While at this secondary school, where he remained until summer, 1858, Schlich kept up his acquaintance with classical subjects. This, however, appears to have been his own choice; the classes his father insisted on his attending were those of mathematics, physics and chemistry. In autumn, 1858, he was sent to the University of Giessen, to increase his mathematical knowledge before joining the Polytechnic College at Karlsruhe, where, from October, 1858, till October, 1859, he studied mechanical engineering under Prof. Raddenbacher. This course over, Schlich matriculated, in October, 1859, as a regular student in the forestry section of the faculty of philosophy, at the University of Giessen.

The care exercised by Pastor Schlich in planning his son's secondary education reflects a respect for the system of education that ensures adequate discipline in one subject before another is taken up, and suggests that the somewhat late commencement and the apparently irregular course of the boy's preparatory training, may have been equally deliberately designed. However this may be, young Schlich was now in a position to profit by the teaching in the section of the faculty he had joined. That section owed the prestige it then enjoyed to the reputation of the celebrated Dr. Gustav Heyer, professor of forestry. The subjects taught formed two groups, an auxiliary and a professional. The auxiliary subjects were mathematics, with surveying; physics, with mechanics; general zoology; general botany; political economy. The professional course, conducted by Heyer himself, dealt with forestry proper in all its branches and



aspects. Schlich found Prof. Heyer the most inspiring teacher he ever heard; Heyer thought Schlich the most brilliant pupil he ever taught.

In 1862 Schlich passed the final University examination in forestry and applied for employment in the Hesse-Darmstadt State Forest Service. Accepted as a probationer, he now commenced the two years course of practical work, and began to prepare for the two distinct state-examinations, which probationership involved. The first year was spent in the headquarters office in Darmstadt; the second in the forest of Viernheim, near Mannheim. Schlich passed the final state-examination in 1865 and in 1866 was appointed Assistant to the Oberförster of Homberg in the Vogelsberg. He seems now to have felt justified in giving its final form to his 'Inaugural Dissertation,' which appears to have been submitted in 1866; it secured for him, in 1867, the degree of Ph.D. This thesis itself suggests that thus early in his career Schlich was already equally interested in forest-production and the utilisation of forest-products: its subject was "the financial aspects of forestry."

Schlich had been but a few months at work under the conservator of the Homberg forest when the outbreak of the Seven Weeks War brought forestry operations to a standstill: the territorial changes insisted upon by the victors when the war ended, deprived Schlich of his appointment. This mishap caused keen disappointment to Schlich's distinguished teacher, who appears to have cherished the hope that Schlich might eventually become his successor in the chair of forestry at Giessen. The accident did not, however, injure Schlich's career; it only diverted his energies. The Government of India in 1866 was still dependent, in large measure, for its supply of forest officers on the transfer of members of other established services, whose professional knowledge had to be acquired by practical experience after appointment; the help of officers who had received professional training or acquired practical experience in forestry prior to selection, could rarely be secured. Among those on whose advice in forestry affairs the Indian Government could then rely, there was one who knew the value of Heyer's judgment and was aware of what had happened to Schlich. As a consequence, Schlich was invited to join the Indian Forest Service. Accepting the invitation, Schlich reported himself at the India Office in December, 1866, and sailed for India early in 1867.

On reaching India Schlich was posted, as an Assistant Conservator of Forests, to Burma, a province in which sound forest economy is especially desirable. Soon after reaching Rangoon in March, 1867, Schlich's attention was directed by his immediate superiors to the combination of forest-rejuvenation with the raising of agricultural crops, a system that had been advocated for Burma a decade earlier but had not been initiated there till 1866. The system is one with which Schlich was already familiar; it had been in use, in the district in which his own practical experience was gained, since the days of the Napoleonic wars. Schlich's duties were so fulfilled as to lead to his advancement, before

1867 closed, to the grade of Deputy Conservator. Three more years of arduous and efficient work in Burma brought him promotion to the grade of Conservator; as such, in 1870, Schlich assumed charge of the forests of Sind.

This transfer to a nearly rainless region, whose only forests owe their existence to the presence of the river Indus, involved the study of problems other than those that need solution in the rain-forests of a monsoon-area like Lower Burma. But the results obtained by Schlich in this new environment proved such as to justify his appointment, towards the end of 1872, as Conservator of Forests in Bengal. His new charge was wide and singularly varied: it included then the forests of Assam, Cachar and Chittagong; those of a section of the Eastern Himalaya with their submontane complement; those of the great Ganges-Brahmaputra deltaic area; the prolongation into the Orissa highlands of the forests of the Eastern Ghats; the north-western extension into Chutia Nagpur of those of the Central Indian plateau. In the face of many difficulties, Schlich proceeded to reorganise the work of a charge that now constitutes three Conservatorships, and was able, during the next six years, to establish a system that was of immediate benefit and has proved of permanent value.

Soon after his transfer to Bengal, Schlich was given an opportunity of bringing to the notice of the Government of India a matter of consequence to Indian forestry as a whole. It was already an established practice to send all young Indian students, recruited for service in the forest departments of particular provinces, to the Government Engineering College at Rurki, there to study for a year. The experience he had gained in three provinces enabled Schlich to state that these young men acquired at Rurki useful knowledge in certain branches of engineering. But he had to add that they had learned little or nothing of forestry. To remedy this defect Schlich recommended, in 1873, an extension of the period of study at Rurki with the addition of forestry classes to the Rurki course. The adoption of these recommendations proved to be impracticable, but the Government of India was so impressed by the cogency of the arguments on which they had been based, that in 1875 sanction was accorded to the provision of forestry instruction at Dehra Dun, and in 1878 a full course of teaching in forestry was inaugurated there.

By 1874, the year in which Schlich married, he had substituted the use of "William" for "Wilhelm" in his private and official correspondence. That year a conference of forest officers, which he attended, assembled in Allahabad. Those present reached a unanimous decision that it was desirable to establish a service magazine; Schlich was invited to carry the resolution into effect. Schlich was able to report to another conference held at Simla a year later the progress he had made, and on July 1, 1875, he issued the opening number of the 'Indian Forester.' He served as honorary editor of the new journal during the first four years of its existence and secured for it, from the outset, the recognised and stable position it has enjoyed ever since. The termination of his editorship was unavoidable. In 1878 his wife had died; he himself now felt the effects of twelve years of unbroken and exacting service in the tropics. He applied for, and was granted, the first and only furlough taken by him while a member of the Indian Forest Service.

On his return to duty in India in 1880, Schlich was appointed Conservator of Forests in the Panjab, only to be transferred, in 1881, to the headquarters of the Government of India to officiate as Inspector-General of Forests. That Government was not then in full touch with the management and exploitation of the provincial forests, though it was known that, in certain cases, particular forests were in danger of being overworked. In 1882, Schlich submitted proposals for centralisation of control of the preparation and execution of working plans. The scheme, which involved the institution of a new forestry branch, under the Inspector-General and with an Assistant Inspector-General in charge, evoked a vigorous protest on the part of at least one provincial administration. But it met with the approval of the Government of India, who confirmed Schlich's appointment as Inspector-General in 1883; it received the sanction of the Secretary of State for India in Council in 1884. Competent authorities agree in regarding this action as one of the most important events in Indian forest history. It was also to be the last outstanding one in Schlich's Indian career. The Secretary of State for India had resolved to effect yet another change in Indian forest policy: in 1885 Schlich received an invitation, acceptance of which involved the close of his service in India.

The India Office had already adopted the policy of providing professional training for all young officers recruited for the forest service. This training had hitherto been obtained on the Continent: the Secretary of State had now reached the conclusion that this training ought to be supplied, as far as possible, in England. In 1885 the India Office did not experience the difficulties that had prevented the Government of India in 1873 from providing Indian students with forestry instruction at an engineering college: the new policy involved the addition of a forestry branch to the Royal Indian Engineering College at Coopers Hill. Schlich had justified Heyer's early estimate of his professional skill and administrative gifts; it was known at the India Office that Heyer had formed a similar estimate of Schlich's capacity as a teacher. An invitation to him to undertake the duties of professor of forestry at Coopers Hill was therefore natural; the success with which, during the next twenty years, Schlich carried out his new duties, amply confirmed the wisdom of the Secretary of State in sending that invitation. While bringing the teaching of his subject at Coopers Hill to a state of high efficiency, Schlich found time to prepare an authoritative 'Manual of Forestry,' in five volumes; three of them written by himself, the other two by the late Mr. W. R. Fisher, who had served under him in India and was his colleague at Coopers Hill. Soon after he took up his duties at Coopers Hill in 1885, Schlich was elected a fellow of the Linnean

Society; in 1886 he married a second time and became a naturalised British subject; in 1891 he was made a C.I.E.; in 1901 he was elected a Fellow of the Royal Society.

In spite of Schlich's successful administration, the India Office arrangements of 1885 did not prove permanent. In 1905 the Secretary of State had to face a difficulty in providing forestry instruction at an engineering college more serious even than those that the Government of India had found insuperable in 1873. It had been decided in Council that the provision of professional training in this country to recruits for the Indian Public Works Department was no longer necessary, and that, as a consequence, the establishment at Coopers Hill must be abolished. The professional training of recruits for the Indian Forest Service was, however, still required; the work conducted by Schlich must be continued somewhere. The University of Oxford offered its hospitality to the Indian Forest School, and upon Schlich, now in his sixty-fifth year, was imposed the task of grafting a modern professional discipline on a venerable academic organisation. The wise judgment of the University authorities, the enlightened generosity of certain Colleges, the tact and knowledge of Schlich, enabled this change to be effected to the satisfaction of the India Office, which took what had been done on its behalf as a matter of course. The arrangements agreed upon proved equally satisfactory to the University, which marked its sense of what Schlich had done by conferring upon him, in 1905, the degree of M.A.

The satisfaction which Schlich's success afforded the University of Oxford and the Secretary of State for India was not shared by other universities. With the notable exception of the University of St. Andrews, where, during the Franco-Prussian War of 1870–71, hospitality and instruction had been given to the recruits for the Indian Forest Service whose professional training on the Continent had been interrupted, the disposition of British universities to help India by teaching forestry had not been conspicuous. The transfer of the Indian Forest School from Coopers Hill to Oxford induced certain sister Universities to organise courses of forestry instruction. So general and intense did this spirit of emulation become that, in 1908, His Majesty's Government threw open the Indian Forest Service to students of any British University approved by the Secretary of State for India. This order, which converted what had been an Indian Forest School into the Oxford School of Forestry, was, at the same time, a recognition of Schlich's influence on the teaching of forestry in this country that, though oblique, was unmistakable; in 1909 Schlich was created K.C.I.E. Already, however, Schlich had won for the Oxford School of Forestry an assured position; in 1911 when Schlich ceased, under Civil Service regulations, to be a public servant, he was appointed University Reader in Forestry and, by a special Decree of Convocation, was given the status of a Professor. This graceful recognition of what he had already done for Oxford only increased his desire to render the University further service.

The power of adapting himself to new conditions which marked every stage of Schlich's career was accompanied by a capacity to realise their possibilities. The order which converted the Indian organisation he had created into an Oxford school, induced a wish that Oxford, like his own old Alma Mater, might one day possess a permanent forestry professorship and establish a forestry school whose studies should serve as an avenue to a degree. With the approval of the University he made an appeal for funds to endow a forestry chair. This appeal met with a generous response from the College of which he was an Honorary Fellow and from many private benefactors, not a few of them old pupils of his own at Coopers Hill. He was actively engaged on this task when in 1913 he was appointed president of the Royal English Arboricultural Society. The outbreak of the Great War in 1914 delayed the realisation of his wishes, but gave him an opportunity of rendering valuable service to the State as a member of the forestry Sub-Committee of the Reconstruction Committee, that began its labours in 1916 and submitted in 1918 a final report, one consequence of which was the formation of the Forestry Commission. At last Schlich had, in 1919, the satisfaction of seeing the acceptance by the University of Oxford of a statute which established a University Chair of Forestry, and of another which placed Forestry on the list of degree subjects. Now in his eightieth year, Schlich felt his academic work had been done; on New Year Day, 1920, he handed over to a successor the duties of his chair, and set to work on a further revision of his already classic 'Manual of Forestry.' By 1922 he had issued a fourth edition of the first volume; in 1925 he was able to complete a fifth edition of the third volume. Soon after the appearance of this, he died, after a brief illness, on September 28, 1925.

A striking personality, of strong character, saturated with knowledge of his special subject and devoted to its advancement, Schlich proved himself in India a skilled and successful administrator; in this country a sound and inspiring teacher. The influence he was able to exercise directly by word and pen, and indirectly, though not less effectively, through the medium of a host of active and eager disciples, is largely responsible for the intelligent appreciation of the importance of a wise forest economy that is slowly but surely gaining ground throughout the British Dominions. The combination of tact, sagacity and "a saving sense of humour" that marked his character, rendered him a reliable adviser and enabled him to overcome difficulties from which many might well have shrunk. By his death our Society has lost a worthy Fellow: his adopted country, a very faithful servant.

D. P.

G. W. LAMPLUGH—1859-1926.

George William Lamplugh, who died on October 9, 1926, was one of the most eminent of the British geologists of the day. He was born at Driffield on April 8, 1859, but at the age of 13 moved to Bridlington. Brought there face to face with the great exhibitions of glacial and Cretaceous strata furnished by the wasting cliffs, he developed a bent for geology which determined his future career. He had actually taken a commercial post, but when the time came for a decision, he definitely put aside the possibilities of affluence in business in favour of a life spent in scientific research.

His earliest work lay among the glacial deposits and already showed a power of bold but controlled speculation which was one of his characteristics. He assiduously examined the sections laid open by the receding cliffs, thereby preserving many a record from total loss. Among the miscellaneous materials of which the glacial drift is composed marine shells are not uncommon. These he collected, paying special attention to their condition, whether fragmentary or perfect, and observing also the occurrence in some of them of a sand, which he recognised as a remnant of the original matrix. He noticed also that the drift contained remains of a fresh-water deposit crowded with shells of *Limnæa peregra*, but drawn out into lenticular strips in the boulder clay. He came to a conclusion, not generally held at the time, that the marine shells were no proof of submergence, but were truly boulders. The great thickness and amassed appearance of the "Bridlington Drift" he attributed to the accumulating power of a huge mass of ice, moving at one time over a soft sea-bottom, at another over the silty bed of a pond.

During these years he was engaged also in an examination of the chalk and underlying strata, more especially of the Speeton Series (Lower Cretaceous). To this work he applied the methods of zonal palæontology, the importance of which British geologists had been slow to recognise. He concluded that the belemnites afforded the best means of zoning the strata, and was not deterred by the fact that the palæontological zones did not always agree with the lithological divisions. Extending his work southwards, he established a correlation of the Speeton Series with the Tealby Series of Lincolnshire, and later contributed an account of these series to a paper, written jointly with Prof. Pavlow, of Moscow, which was of European significance.

This early work had marked out Lamplugh as a geologist of promise, and when in 1892 he decided to abandon business, he received an appointment on the Geological Survey. The first duty assigned to him was the geological surveying of the Isle of Man, a task which demanded the highest qualities in every

G. W. Lamplugh

branch of geology. His experience of glacial deposits stood him in good stead, but he had to deal with problems in the older and newer Palæozoic rocks, in petrology and vein-formations, all of which were new to him. He completed the work in five years and, in addition to the map, produced a memoir which not only furnishes a detailed account of the island, but takes rank as a classic in modern geology.

In 1901 he was put in charge of the Irish branch of the Geological Survey, and resided in Dublin until that branch was transferred to an Irish department in 1905. He himself took part in the surveying of the country round Dublin, Belfast, Cork and Limerick, concentrating attention more especially upon the glacial deposits. On his return to England he superintended the surveying of the Midland and North Wales districts, but the later years of his official life were spent in re-examination of the Wealden area. Here the exploration, by boring, of the newly discovered coal-field was in progress. The object of the bore-holes was to prove the extent and character of the coal-field, and the details of the overlying Secondary rocks received scant attention. Some reluctance, moreover, was shown by the explorers to make known their results while the value of the coal-field was still in question. By tact and perseverance Lamplugh not only secured the records, but interpreted many which would otherwise have been unintelligible or misleading. His account of these borings, coupled with a palæontological contribution by Dr. Kitchin, threw a new light on the Secondary geology of Southern England. In 1914 he was appointed Assistant Director for England and Wales, a post which he occupied until his retirement in 1920.

The interest in the Lower Cretaceous rocks which had been roused in Yorkshire was kept alive by the work in the Wealden area. He had also worked unofficially in an intervening district in Bedfordshire. Near Leighton Buzzard he had watched for some years the exposures of a fossiliferous band below the Gault. The fossils were not those usually found at that horizon, and were in fact claimed by palæontologists as characteristic of a horizon above the Gault. It was argued that the strata must have been inverted. Lamplugh, in his last paper to the Geological Society, showed that the band had been met with over a considerable area, and that in every successive exposure it had been found to occupy the same position below the Gault. The suggestion of inversion he regarded as untenable, and did not consider the palæontological evidence in favour of it to be conclusive. It was his intention to continue his work on Lower Cretaceous rocks after his retirement, but failing health soon rendered this impossible.

Lamplugh seized every opportunity of travelling. He visited the Eastern and Central States of America, Vancouver, Alaska and the Mexican Border. In 1905, at the request of the Council of the British Association, he made a systematic examination of the gorge of the Zambesi below the falls. The chasm into which the river plunges had been attributed to a gaping crack in the crust of the earth, but Molyneux had argued that it was the work of fluviatile erosion. Lamplugh was able to prove that the chasms and the gorge were the work of the river gnawing its way back into the Continent, and taking advantage of every crack, vein, or other plane of weakness in the rocks. In 1910 he visited Spitzbergen and there saw in operation the process of accumulation of a boulder clay which he had pictured many years before. In 1914 he accompanied the British Association to Australia.

Lamplugh was elected to the Royal Society in 1905 and served on the Council in 1914–16. Elected to the Geological Society in 1890, he served several times on the Council and was President in 1918–20. He presided over Section C of the British Association in 1906, and was Past-President of the Yorkshire Naturalists' Union, the Hull Geological Society, and the Hertfordshire Natural History Society, and was an honorary member of several other societies. He received from the Geological Society a moiety of the Lyell Fund in 1891, the Bigsby Medal in 1901, and the Wollaston Medal in 1925.

His work was characterised by boldness and originality of thought, controlled by sound judgment, and founded on exhaustive observations in the field. Habitually he devoted prolonged consideration to a subject before forming an opinion, and in dealing with speculative matters never exceeded the limits imposed by his evidence. He read widely and with discrimination, and in his own writings took infinite pains to secure the exact form of words by which to express his meaning. We have lost in him a sound geologist and a lovable friend.

A. S.

George Adami

JOHN GEORGE ADAMI—1862–1926.

ULTIMATELY of Italian descent, Adami was born in Manchester on January 12. 1862. He was brought up at the Grammar School and Owens College and in 1880 went to Cambridge as a scholar of Christ's College. He got firsts in both parts of the natural sciences tripos, with physiology as his subject in part II. worked abroad in Heidenhain's laboratory at the circulation in the frog's kidney, went back to Manchester to finish his clinical work, and in 1888 became Roy's demonstrator at Cambridge. They worked together till 1892 when Adami was appointed first professor of pathology in McGill University, Montreal, and here he spent the greater part of his active life. During the War he was busy in Europe with the Canadian Medical Corps. When it was over in 1919 he gave up his chair and became Vice-Chancellor of the University of Liverpool where he died on August 29, 1926. He was elected a Fellow of the Society in 1905 and served on the Council in 1915–17.

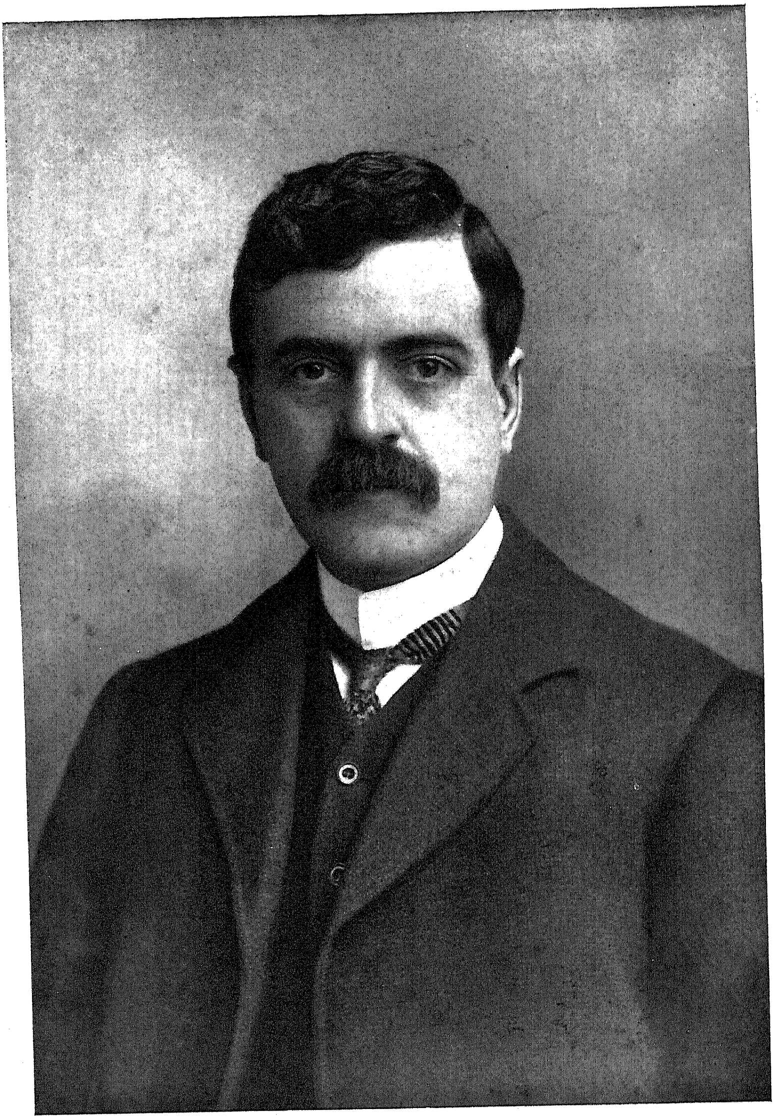
Adami went to Cambridge at a stimulating period in the development of biology in that University and he was fortunate in his contemporaries. His early training in physiology found its natural outlet when he became associated with Roy, who had learned from Cohnheim how physiological methods might be exploited in the study of functional pathology. Their special interest was the cardio-vascular system and its explanation by graphic records, and its culmination will be found in their classical paper on "The Physiology and Pathology of the Mammalian Heart" in the 'Philosophical Transactions' (1893; vol. clxxxiii, p. 199) with subsidiary articles in the medical journals on heart strain and similar subjects.

After he went to Canada, Adami was not much given to doing laboratory work on his own account. He had a fresh department to bring into working order and to establish as an integral part both of the University and of the Royal Victoria Hospital, and his practical activities are represented in the literature mostly by the abundant papers of his colleagues and assistants, in many of which he obviously played an activating part. But his studious habits resulted in two notable works. He wrote the article on "Inflammation" for Allbutt's 'System of Medicine' (1896, vol. 1, p. 54) which was subsequently expanded and published separately, reaching a fourth edition in 1909. It is an admirable exposition of the argument that inflammation is a topic of general biology, of much wider significance than its daily illustrations in medicine and surgery would suggest, and it has had considerable influence in guiding the thoughts of students along broad and sound lines. By 1909 he had extended the general ideas of 'Inflammation' over a wider basis and published the first volume of his 'Principles of Pathology.' In this he seeks not so much to give a presentation of pathological data (which had been done well enough and quite often enough already, and which he did again for some parts of the subject in the second volume of the 'Principles,' quite an ordinary book) as to put them together and analyse them in an orderly manner, and to bring them into relation with other branches of biological study. It is an attempt to develop pathology as a science, and if any one to-day dwells overmuch on its obvious shortcomings it is well to remember that it was in its day highly original in conception as well as execution. It was also before its time, for after a second edition in 1910 its brief popularity faded away and it is now almost forgotten; pathologists still spend most of their time over special instances and are not much interested in general propositions.

Adami indeed was at heart a biologist, and his twelve years at Cambridge had taught him that pathology could be most fruitfully pursued as a branch of biology rather than as an appendage to practical medicine. As far back as 1892 he wrote on the variability of bacteria and the evolution of races among them, and he devoted his Croonian Lectures to the Royal College of Physicians of London, in 1917, to inheritance and adaptation as illustrated by pathology. His classification of tumours, which survives with a strange popularity, was made on an embryological basis, and his early training shows up wherever there is anything to reflect it. Towards the end he was sadly disappointed that he had not been able to establish "general" pathology more firmly. He writes of "fighting a losing cause" and finding "no sympathy for pathology that was not essentially applied to medicine and surgery." Perhaps to some extent his own interests were divided. Biologist as he was, it was impossible for a man of his vivid human interests not to feel keenly the individual claims of the sick people with whom he necessarily came in contact, and he wrote and spoke abundantly on medical subjects. Perhaps too he succeeded better than he thought; the admission of pathology as a subject for Part II of the Natural Sciences tripos at Cambridge is a beginning after his own heart.

He was a genial and very friendly man, and a good Christian.

A. E. B.



Benjamin Moore.

BENJAMIN MOORE—1867-1922.

Benjamin Moore devoted his maturer years to Biochemistry and came to occupy, with distinction, two important Chairs founded to advance that subject. His early training, however, was intended to prepare him for the calling of an engineer. A developing taste for pure science, and especially for experimental physics, led him after graduation to alter his choice of a career, while the influence of Wilhelm Ostwald a little later directed his mind more particularly to the rapidly growing subject of physical chemistry. The application of physico-chemical methods to biological problems next attracted him, and he discovered finally that in this sphere was his real vocation. His early experiences and training could not fail, however, to affect his work and thought when he turned to biological studies. To these he brought great enthusiasm and abundant enterprise.

Although his earlier experimental studies touched in turn very many isolated aspects of physiology and biochemistry, in his latest years he became rather impatient of detail. He determined to deal, so far as might be possible, with the more fundamental problems of biology. He speculated boldly, and believed in the courageous application of experimental methods to the solution even of such great problems as the origin of life. He was indeed always preoccupied with the broader aspect of things; with the riddle of the physical universe as a whole; with the present and future welfare of the human race as a whole. If as a scientific worker he sometimes overvalued the experimental material on which his bold conceptions were based, he nevertheless often proved himself—when his enthusiasms were less involved—to be an acute critic, alike of his own work and that of others. Many of his papers reveal flashes of real inspiration, and, particularly when discussing physico-chemical aspects of the living cell, he often incidentally advanced ideas which, while wholly original at the time, have since been confirmed experimentally and accepted by others. The progress of biochemistry during the first twenty years of this century owed much to his stimulating publications and to his personal influence over his colleagues and pupils. To the exact data of the science his contributions—save in certain limited fields—were not numerous. He lacked the patience, or, perhaps, the stolidity of mind which makes attractive the laborious details of research.

Moore was born in 1867. He was educated at Queen's College, Belfast (now the Queen's University), where he graduated as a Bachelor of Engineering. He then proceeded to Leipzig and worked in Ostwald's laboratory, carrying out a research on the velocity of crystallisation in super-cooled phenol. From Leipzig he went to University College, London, to study physiology under Prof. (now Sir Edward) Sharpey Schafer. He was associated with Schafer in researches upon the spleen and the salivary glands, and from the University College laboratories he published work, carried out alone or in conjunction with juniors, upon emulsification, upon the chemistry of the suprarenal cortex, and upon the pharmacology of piperidine, conine, and nicotine. To the first volume of Schafer's Text-Book of Physiology, which appeared in 1899, he contributed a critical and very admirable section on the chemistry of digestion, which corrected current errors and stimulated fresh researches.

From University College he went to Yale to occupy the post of Associate-Professor of Physiology, and from there, conjointly with pupils, he published work upon the origin of lactose, further researches into the functions of the suprarenals, and an important paper upon osmotic properties of colloidal solutions, a subject to which he afterwards paid much attention. After a relatively brief stay in the United States he returned to England and became Lecturer on Physiology at the Medical School of Charing Cross Hospital. During this period of his career he became himself medically qualified.

In 1902 he was elected to the Johnston Chair of Biochemistry at the University of Liverpool, and at once became very active in prosecuting and stimulating research in his subject. His labours while holding this chair covered a very wide ground. He published, chiefly in conjunction with colleagues and pupils, a quick succession of papers, dealing with such diverse subjects as the acid-base equilibrium in the blood of patients with malignant disease, the action of anæsthetics, the action of light and of varying oxygen tensions upon micro-organisms, the oxidising mechanisms of the tissues, the anti-neuritic vitamine, the toxic action of metals, the phenomena of hæmolysis, and other matters.

Of his researches carried out at Liverpool those which bore upon the equilibrium between the living cell and its environment seem to have been the most important to his own lines of thought. Three papers: one published conjointly with H. E. Roaf, on the measurement of osmotic pressure in colloid solutions (1907), with another on the osmotic equilibrium of red blood corpuscles (1908), and a third, with A. D. Bigland, dealing with the nature of colloidal adsorption (1911), together contain the chief experimental basis for his thought and teaching concerning the above fundamental subject. He rejected the view that a membrane with specific permeabilities determines the heterogeneous equilibria which obtain between the cell interior and surrounding media. He attributed them rather to the formation within the cell of reversible chemico-adsorptive associations between colloids and crystalloids, involving probably the activity of residual valencies. In the first of the three papers just mentioned Moore put forward many suggestive and enlightened views. It is clear, for instance, that he possessed at this time a fairly definite conception of the membrane equilibrium which, four years later, was quantitatively studied and clearly defined by Donnan.

In 1906, in co-operation with Edward Whitley, Moore founded the Biochemical

Journal, editing it until 1912, when its control was taken over by the Biochemical Society. The existence of this Journal has been of signal service to English biochemistry. In 1911 he published a small book called "The Dawn of the Health Age," which contained suggestions for a State Medical Service. He was always much concerned with the welfare of the poor, and this book closes with the following paragraph:—

> "We are one species, we human kind, and one class cannot safely or permanently rise or fall by itself; we must take the others up or be dragged down ourselves along with them."

In 1912 he was elected a Fellow of the Royal Society. In 1914 he left the Liverpool Chair to join the staff of the National Institute for Medical Research (now so-called), at Hampstead, under the control of the Medical Research Council. The immediate advent of the War diverted his energies, as those of all his new colleagues, into special channels. He engaged, for instance, in intensive studies of TNT poisoning and miners' phthisis. His own views concerning their prevention and cure did not always conform to those of the majority, and he displayed a characteristic fighting spirit in defending them; always with the welfare of the workers in mind.

In 1920 he was called to the Chair of Biochemistry at Oxford, founded on the initiative of his intimate friend and former colleague, Mr. Whitley. Relieved from the preoccupations of the War, he had turned eagerly to pure science, and when he went to Oxford his mind was full of the problem of the origin of life. His experimental work became an intensive endeavour to throw light upon it. The work he accomplished upon these lines is at least highly interesting, though some of the experiments involved—for instance, those which he believed brought proof that marine and fresh-water algæ can fix atmospheric nitrogen—were not above criticism. Had he lived, however, his fearless enthusiasm and energy might have carried him far, even on this ambitious quest. He died on May 3, 1922, from sequelæ following on an attack of influenza. Lack of health for some little time before had weakened his resistance.

Only a year before his death he published a book on Biochemistry. This is in no sense a general text-book. It deals in the main only with those aspects of the subject which had special interest for its author. It is indeed essentially a summary of his own work, thought and views. It is fortunate that he lived to write it, for it makes clear the outlook to which he had attained, and is the scientific testament of an exceptional and most interesting personality.

Moore's Irish origin made him a rebel against orthodoxy and authority, in science no less than in social affairs. It may also have helped to make him what by common consent he was—an inspiring teacher, a delightful colleague, and a most warm and faithful friend.

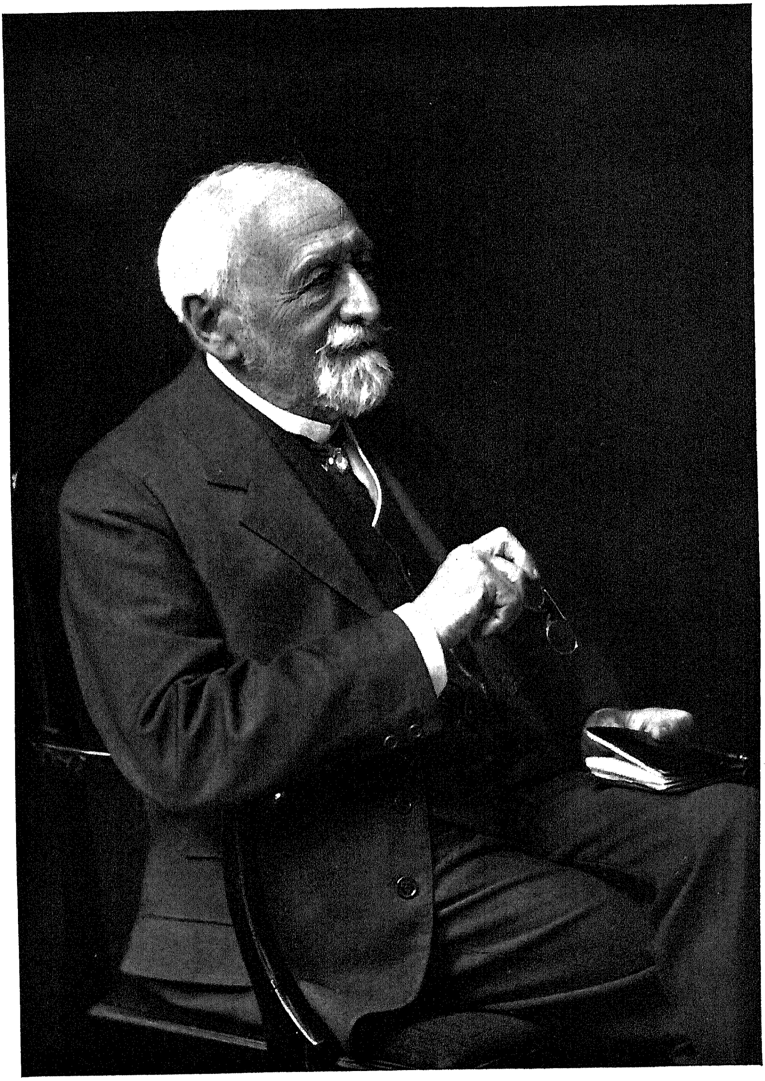
F. G. H.

SIR THOMAS CLIFFORD ALLBUTT—1836-1925.

The Right Honourable Sir Thomas Clifford Allbutt, who died very suddenly on February 22, 1925, was born on July 20, 1836, and was the only son of the Rev. Thomas Allbutt, Vicar of Dewsbury, Yorkshire. From childhood he was interested in natural history, and he acquired these tastes from his father, who was a friend of Charles Waterton, and from Archdeacon Hey, under whose direction he was educated at St. Peter's School, York. He entered Gonville and Caius College, Cambridge, in 1856, and had a distinguished career as an undergraduate, obtaining a classical scholarship and a first-class (the only one) in the Natural Science Tripos of 1860. He studied medicine at St. George's Hospital, where he was more especially a pupil of Bence Jones and of Lockhart Clarke, and took the M.B. Cambridge in 1860, and the M.D. in 1868; and he also followed the practice of Trousseau in Paris for a time. From 1862 to 1889 he practised as a physician in Leeds, and from 1864 to 1884 served as physician to the Royal Infirmary, Leeds, and as a teacher in the Medical School.

Notwithstanding the claims of an extremely large consulting practice in the North of England, Allbutt, from the commencement of his career at Leeds, found time for the prosecution of much original work in medicine and especially in clinical medicine, and much of this has taken a permanent place in the advancement of knowledge. Whilst still a student he had become interested in diseases of the arteries, and throughout his long life he pursued this subject, and even in the last few weeks of his life he completed a short monograph on arterio-sclerosis, containing a summary of his mature conclusions on this subject, and as early as 1868 he had described syphilitic disease of the cerebral vessels. Allbutt, apart from his principal work on the cardio-vascular system, made two outstanding contributions to the advancement of medicine. He invented the short clinical thermometer that is now in constant daily use in the practice of medicine, and in 1871 he published a work on the use of the ophthalmoscope, not only in diseases of the nervous system, but also in disease generally. The general use of the ophthalmoscope in medicine that has proved of so much value is largely due to these writings of Allbutt, and his advocacy of the use of the thermometer and of the ophthalmoscope affords good instances of his scientific method in the adoption of instruments of precision in the routine practice of clinical medicine.

Although Allbutt's interests in medicine were wide, and he wrote on many diseases, more especially on affections of the stomach and on some so-called functional disorders, his main work was that devoted to cardio-vascular disease, and more especially to angina pectoris and to morbidly high blood



pressure, to which he gave the name of hyperpiesia. He published in 1915 his principal work dealing with these subjects under the title of "Diseases of the Arteries," including angina pectoris. This is universally recognised as an outstanding contribution to a difficult subject, and his conclusions have in the main been generally accepted.

In 1889 he became, after relinquishing practice as a physician, a Commissioner in Lunacy, and acted in this capacity for some three years, but in 1892 he accepted the Regius Professorship of Physic in the University of Cambridge, and fulfilled all the duties of the Chair until his death, his vigour and mental alertness being such that notwithstanding his great age he gave clinical lectures up to the very end. His life as Regius Professor was a very full one, as he constantly gave addresses which were always characterised by an exceedingly broad and comprehensive outlook, and were remarkable for their gracefulness and polished style. During this period he brought out, as Editor, the well-known "System of Medicine" (1896–99), which for many years was the standard and most authoritative work on medicine in the English language.

Allbutt was not only a very distinguished clinical physician with a keen appreciation of the value of scientific method, but he was also a scholar, and in many respects resembled the old type of scholar-physician of a bygone time. He was keenly interested in the medicine of the Ancients, and he did much to revive in this country the study of the history of medicine, writing largely on this subject. In 1921 he published his work on "Greek Medicine in Rome," a work based on the Fitzpatrick Lectures delivered by him before the Royal College of Physicians in 1909 and 1910. Previously in 1901 he had delivered the Harveian Oration on "Science and Mediæval Thought," and in 1904 at St. Louis he had given an address on the "Historical Relations of Medicine and Surgery to the end of the Sixteenth Century."

Allbutt served on several Departmental Committees, more especially those on National Insurance and on Trade Diseases. With reference to the latter subject he was instrumental in the formation of the Glassmakers' Cataract Committee of the Royal Society, under whose auspices the question of glassmakers' cataract was investigated. This led, amongst other results, to the production of what is now known as Crookes's glass, thanks to the work done by the late Sir William Crookes when a member of the Committee.

Allbutt was elected a Fellow of the Royal Society in 1880, and served on the Council 1896–98, and again in 1914–16, when he was a Vice-President. He took an active share in the work of the Society, serving not only on the Glassworkers' Cataract Committee, but also on the Tropical Diseases Committee when the latter Committee was supervising the long series of investigations into malaria, Malta fever, and sleeping sickness.

He was a Fellow of Caius College, Cambridge, and received many academic and professional honours. He was created K.C.B. in 1907, and, much to the gratification of the medical profession, was made a Privy Councillor in 1920. During his life in Leeds he was well acquainted with George Eliot, and it was generally believed that she modelled the character of Lydgate in "Middlemarch" to some extent on him.

Allbutt was an outstanding figure in the profession of medicine in this country, admired for his scholarly attainments, his professional knowledge and scientific distinction and respected for the courageous character of his utterances when the need arose for an expression of opinion on matters of professional importance or interest.

Allbutt married Susan, daughter of the late Thomas England, of Headingley. She survives him, but there was no issue.

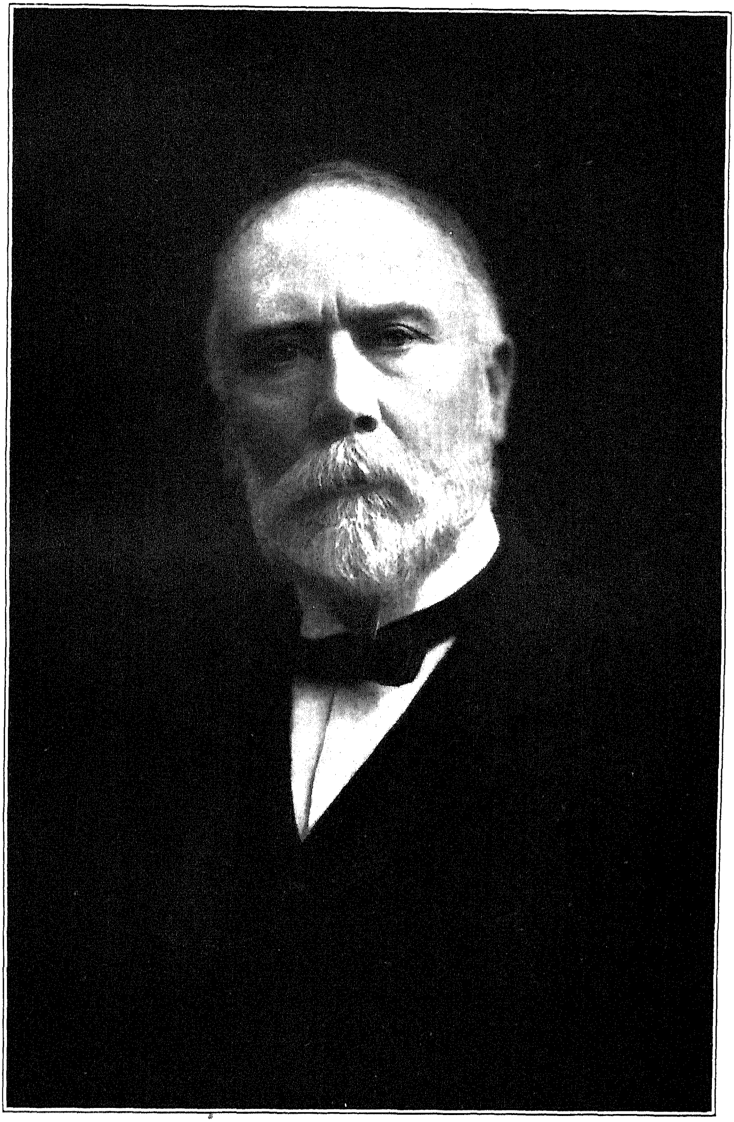
J. R. B.

SIR JAMES MACKENZIE—1853-1925.

Sir James Mackenzie was born at the farm of Pickstonhill, in the parish of Scone, two miles from the ancient city of Perth, on April 12th, 1853, and died in London on June 26th, 1925, being thus in his 72nd year. The disorder from which he died was that which he did so much to elucidate—namely, heart-failure, this, in his case being attended by *angina pectoris*.

He was educated in the Academy, Perth, and in his 15th year became apprenticed to a chemist and druggist in that town, in which situation he was brought into touch with medical men and had his thoughts turned towards medicine as a profession. He became a student of medicine in the University of Edinburgh in the autumn of 1874, and obtained the degrees of M.B. and C.M. of that University in the spring of 1878, being then twenty-five years of age. After holding a house-residentship in the Royal Infirmary, Edinburgh, for six months, he joined, early in 1879, Dr. William Briggs and Dr. John Brown who carried on a medical practice in Burnley, Lancashire. In due time he became chief partner and practised in that busy manufacturing town for over 27 years—when, at the end of 1907, being then in his 54th year, he moved to London with a view to taking up consulting practice. During his practice in Burnley he built up a reputation which gave him a place among the first physicians of his time.

His career as a student gave no indication of the power he developed at



J Mackenzie

Burnley as an exact observer and trained thinker, nor of the burning resolution which afterwards became manifest in him, to do his utmost to advance the theory and practice of medicine. In his earlier years in Burnley he began a systematic enquiry into the origin and nature of the symptoms which accompany disease, particularly into the manifestations of pain, keeping exact records of all those cases where visceral disorders were accompanied by areas of disturbed sensation in the skin and deeper tissues of the body wall. In 1892 he published "A Contribution to the Study of Sensory Symptoms Associated with Visceral Disease" ('Manchester Medical Chronicle,' 1892, vol. 16, p. 293) in which many new facts and new interpretations were brought forward—interpretations which later observation has justified. The belief that all referred pains arise from a disorder or disturbance of the normal reflex actions of the body engaged his attention more and more. In 1906 he published a further contribution to the subject under the title "The Meaning and Mechanism of Visceral Pain" ('Brit. Med. Journ.,' vol. 1, pp. 1449, 1523 (1906)). Later still in "Symptomatology" (1923) and in articles contributed to the 'Brit. Medical Journal,' 1924 ("A New Outlook in Medicine") he sought to extend this conception to embrace the whole field of medical diagnosis.

Sir James Mackenzie's most important contributions to medical knowledge relate to irregular actions of the heart. Very soon after his arrival in Burnley he attended a young pregnant woman who suffered from valvular disease of the heart. She died, after delivery, from heart failure. Wishing to discover the kind of heart lesion which rendered child-bearing dangerous, he began a systematic investigation of the action of the heart in pregnant women, and resolved to make a graphic record of the pulsations which so often became manifest in the veins of their neck. In this way he was led to study the "jugular pulse" which at the time he made his first records (1882) was supposed to be due to reflux waves from the right ventricle. Combining simultaneous tracings from jugular vein, radial artery and heart's apex, he was able to give a more detailed and exact analysis of the jugular pulse than had been given before, and to show that by such triple tracings a new light was thrown on arrhythmias of the heart. He kept graphic records of his cases year after year and learned that in some of them one form of irregularity might suddenly change, the change being indicated by the disappearance from the jugular tracing of the waves which he believed were caused by the contraction of the right auricle. He inferred that, in such cases, the auricles had become suddenly paralysed and gave the name "auricular paralysis" to this state, which he proceeded to show was of frequent occurrence among his patients.

His first paper on this subject was published in 1893 under the title "The Venous and Liver Pulses and Arrhythmic Contractions of the Cardiac Cavities," ('Journ of Path. and Bact.,' vol. 1, p. 53 (1893); vol. 2, pp. 84, 273 (1894)), and his records and knowledge continuing to increase he prepared and published them in book form under the title 'The Study of the Pulse, Arterial, Venous and Hepatic, and of the Movements of the Heart' (Edinburgh, 1902). This is his most important book, containing a wealth of original observation. In 1906 the late Prof. A. R. Cushny visited Burnley and saw some of Mackenzie's cases of "Auricular paralysis," and suggested to him then, or soon after, that the condition was that known to physiologists as "auricular fibrillation" —an explanation which was accepted by Mackenzie. In 1909 Sir Thomas Lewis applied the string-galvanometer to the analysis of arrhythmias of the heart, and in 1912 demonstrated the exact nature of the lesion which Mackenzie originally supposed to be due to a paralysis of the auricles.*

After his arrival in London, at the end of 1907, he completed a text book on "Diseases of the Heart" which speedily passed through three editions, and was translated into French and German. In 1908 he was appointed physician to the Mount Vernon Hospital for Diseases of the Chest, and in 1913 became physician to the department of Cardiology which had just been constituted at the London Hospital. In 1915 he was elected a Fellow of this Society, became physician to H.M. the King in Scotland, and had the honour of Knighthood bestowed on him.

At first patients came to him slowly, but afterwards his life in London became a very busy and prosperous one; physicians and patients came to him from all parts of the world. In 1918, having reached his 66th year and finding that his means had become equal to the modest needs of his household, he resolved to retire from practice. His choice fell upon St. Andrews, but if he anticipated to find rest and recreation there he had not reckoned with his own arduous nature for he was a man who had an intense desire for the welfare and progress of medicine. In 1919 he set up at St. Andrews "An Institute for Clinical Research," the main object being the study of disease in its earliest manifestations. He enrolled in the service of this Institute, now known by his name, the medical professoriate and practitioners of the town and district, and set aside every fee which he earned after his arrival in St. Andrews, amounting ultimately to £10,000, for the maintenance of the Institute. Soon after his arrival in St. Andrews the symptoms of his own disorder became more manifest, and at the end of 1924, in search of a milder climate, he returned with his family to London where he died on January 26, 1925.

A. K.

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* Those who desire further information concerning the steps which led to the recognition of "auricular flutter" and "auricular fibrillation" as clinical entities will find it in lectures by Sir Thomas Lewis ('Lancet,' 1921, vol. 1, p. 785), and by Sir James Mackenzie ('Brit. Med. Journ.,' 1922, vol. 2, p. 71).

J S Gamble.

FREDERICK WILLIAM GAMBLE—1869-1926.

Frederick William Gamble, who died on September 14 last year, was a distinguished zoologist and a remarkably fine teacher. Born on July 13, 1869, he lost his father, Mr. William Gamble, who had been engaged in the cotton trade in Manchester, when he was very young, and his upbringing was, therefore, mainly under the direction of his mother who was the daughter of Mr. John Napier, also of Manchester.

As a Manchester lad it was only natural that he should be sent to the famous Grammar School in that city, where he must have been a contemporary of the present Lord Chief Justice and other boys who have since reached positions of high distinction. At school he became an enthusiastic member of the Natural History Society, and eagerly looked forward during term time to his release from the gloomy surroundings of the school in order that he might add to his collections of butterflies and moths in the Lake district and the coast of Wales, where he used to spend many months of his vacations. He did not specially distinguish himself as a scholar in a school where the classical and mathematical traditions prevailed and no interest was taken in boys with tastes for Natural History.

On leaving school he went to the Owens College with the intention of studying engineering, but finding that the work in that department was not to his taste he soon decided to read for a degree in science, with zoology as one of the subjects. There can be no doubt that Gamble was very strongly influenced by the vivid and stimulating teaching of Milnes Marshall. His juvenile enthusiasm for natural history matured under Marshall's guidance into a cautious but determined passion for scientific study and research. He was the first student to gain the distinction of a First Class in the Honours School of Zoology of the Victoria University, and, encouraged by this success, he decided to adopt a scientific career and to extend his experience by a period of study with Prof. Leuckart in Leipzig.

From Leipzig he was recalled by Marshall to take up the post of Junior Demonstrator in Zoology in the Owens College, and about this time he was invited to contribute the articles on the "Platyelmia" for the 'Cambridge Natural History.' For this task, although young and inexperienced, Gamble proved himself well fitted. The knowledge that he had gained of the parasitic worms in Leipzig, and his wide and critical study of the literature of the group, enabled him to produce a contribution to the Cambridge volumes which was greatly appreciated by the students of zoology of that period. The first original papers from his pen appeared in 1892 and 1893—one on some rare British Nudibranchs, and the other a contribution to our knowledge of the British

Marine Turbellaria. They were mainly descriptive and systematic in character, but they gave some indications of the tendency of his mind towards experimental zoology.

On the retirement, in 1895, of Dr. Hurst from the Senior Demonstratorship Gamble gained his promotion, and he retained this post until 1909 when he was appointed Professor of Zoology in the University of Birmingham. It was soon after he became senior demonstrator that the University authorities introduced into the syllabus for the 1st M.B. examination the Lug-worm (*Arenicola marina*), but, finding that the published accounts of this type were superficial and in some respects inaccurate, Gamble and his colleague Ashworth undertook a very thorough investigation of its anatomy, the results of which were published in the *Quarterly Journal of Microscopical Science* in 1898.

Leaving the completion of the work on Arenicola in the able hands of his colleague, Gamble next turned his attention to the remarkable colour changes of the phantom shrimp *Hippolyte*, which he had observed during a visit to the marine biological station at Piel in Lancashire, and, forming a new partnership with his botanical colleague F. W. (now Sir Frederick) Keeble, he threw himself with all his energy and insight into the new investigation. In the very interesting paper that the joint authors published in 1900 the various colour changes to which these Crustacea are subject, under different conditions of intensity of light and colour of the background, were described, and the discovery of a remarkable nocturnal phase, in which all the coloured varieties assume a blue tint, was announced.

The work on *Hippolyte* led to further investigations on the chromatophore systems and the physiology of colour change in other Crustacea, Gamble undertaking the morphological side and his partner the physiological side of the problems. The results of these researches, which were carried on partly in Keeble's private laboratory at Tregastel on the coast of Normandy and partly in the Universities, were published in two papers in the *Philosophical Transactions of the Royal Society* (1904 and 1906).

In these papers it was shown that colour pattern in the Mysidacea is due to a primary system of chromatophores, whereas in the higher Decapoda there is a secondary, or in the case of Hippolyte a tertiary, system of chromatophores which is responsible for the colour pattern. The results of a further investigation of the nocturnal blue tints were described, and an account given of the relation of the mobile fats to the chromatophores under the influence of light. Four years later Gamble published a paper on the results that he had obtained in the Port Erin Laboratory on colour change in the young Wrasse (*Crenilabrus*) and in *Hippolyte*. He discovered that, when exposed to the light transmitted through different coloured seaweeds, they assumed the complementary colour to that of the seaweed.

While the investigations on colour change were still in progress Gamble and his partner undertook researches on the chlorophyll-bearing organisms of the little marine flat-worm Convoluta. After observing the tidal appearance and disappearance of the Convoluta at the surface of the sand, they discovered that the green organisms can under certain circumstances escape from the body as an Infusorian with four flagella, allied to the genus Carteria, and proved that its presence is due to the infection of the Convoluta at an early stage of its life-history. The interesting hypothesis they put forward, that the association of the two organisms was due to the nitrogen requirements of the flagellate organism, and that it had led to the loss of the excretory as well as the normal digestive organs of the Convoluta, was supported by strong evidence and generally accepted. There can be little doubt that the two papers they published on this subject in 1904 and 1907 constitute one of the most important contributions to our knowledge of Symbiosis that have appeared in recent times.

All the time that Gamble was engaged in the production of these important researches, he was carrying on the arduous duties of his post as Senior Demonstrator and Lecturer in Zoology in the University of Manchester with extraordinary tact and industry. His power of gaining the mastery of a subject for his teaching is indicated by his excellent account of the Radiolaria in Lankester's *Treatise on Zoology*, and of his method of presenting biological problems to a popular or elementary audience by his little book called " Animal Life," published by Smith Elder in 1908.

He was elected a Fellow of the Royal Society in 1907, and two years later was appointed to the Professorship of Zoology in the University of Birmingham. In this new post he soon showed his powers as a teacher, administrator and director of research, and his influence was seen in the published researches of several young zoologists he had trained and encouraged. The gain to Birmingham by this appointment was a severe loss to Manchester, where he had endeared himself to his colleagues by his brilliant work and many other sterling qualities ; and Birmingham soon learned to value him as Manchester had done. A colleague of his wrote after his death, " He will be a great loss to the (Birmingham) University for, from my long connection with the academic world, I can say, without exaggeration, that it is very rare to find in a professor the combination of qualities, intellectual, social and spiritual which he possessed and which endeared him to colleagues and students."

To those who were intimately acquainted with his life and work his wonderful and accurate knowledge of many branches of zoology was well known, but owing to a singular modesty of manner, amounting almost to shyness at times, it was not apparent to those who met him only occasionally. From his published writings it might be thought that he was essentially an " invertebrate " man, but for many years he was in charge of the teaching of the comparative anatomy of the vertebrata to the senior students in Manchester, and his knowledge of the higher animals was accurate and extensive. His interest in the more philosophical problems of the science, and the well-balanced judgment he brought to bear upon them are indicated in the remarkable address he gave in Toronto, as President of Section D of the British Association.

It is rare to find a man of such wide scientific accomplishments, and in these days of high specialisation his loss will be severely felt.

He married in 1904, Ellen, daughter of the late Rev. J. M. Bamford, of Arnside, who survives him. He left no children.

S. J. H.

H. B. GUPPY—1854–1926.

Dr. Henry Brougham Guppy was born in 1854 at Falmouth. He was educated at King's School, Sherborne, Queen's College, Birmingham, St. Bartholomew's Hospital, London, and Edinburgh University. As surgeon in Her Majesty's Navy (1876–1885), he served on the China and Japan station, on the "Hornet," from 1877–80, and in 1878 visited the Korean Archipelago, where he made geological and meteorological observations. From 1881–1884 he served on H.M.S. "Lark," commissioned for survey work in the Western Pacific, and studied the ethnology, geology and natural history of the Solomon Islands.

In his book on the Solomon Islands (1887) he describes the result of his investigations on the geology of the group, from which he infers that the coral reefs were formed in a region of elevation not of subsidence, that they are of moderate thickness, and are on the site of anciently submerged volcanic peaks. He declares himself an adherent of the theory of the formation of coral islands advanced by John Murray, and in opposition to the view elaborated by Charles Darwin, at any rate so far as concerned the Solomon Island group. When, at the instance of Murray, he visited the Cocos-Keeling Islands, in 1888, spending ten weeks on that classic ground, his careful observations confirmed him in his view.

But the problem of plant-dispersal by means of water was his chief interest, and formed the subject of his most important contributions to science. A paper on "The River Thames as an Agent in Plant Dispersal," read before the Linnean Society in 1892 (*Journ. Linn. Soc., Bot.*, vol. 29, p. 333), was a careful study of the duration of the floating capacity and viability of the numerous plant-species represented by fruits and seeds in the river drift. Then came

H. B. Guppy

some years of work in the Pacific—a year in Hawaii, and two years and three months in Fiji—the results of which appeared in his book, "Observations of a Naturalist in the Pacific between 1896 and 1899." Vol. I (1903), a description of the leading physical and geological characters of Vanua Levu, envisages this and the neighbouring island, Viti Levu, as rising from a submarine platform built up from the deeper ocean floor by submarine lava-flows and associated deposits; the two islands having been formed during a long period of emergence by the union of a number of large and small islands, the product of submarine eruptions. The second volume, *Plant Dispersal* (1906), deals with this subject in relation to the littoral floras of the Pacific Islands, and the part played by ocean currents in transporting fruits and seeds. From 1906 to 1914 he carried out similar investigations in the West Indies and the Azores, the results of which are embodied in his *Plants, Seeds, and Currents in the West Indies and the Azores* (1917). These two books on plant-dispersal contain a great wealth of facts, the results of careful observation and experiment, and form invaluable text-books for the student of plant-distribution and island floras. In connection with his studies on dispersal, Guppy made a series of experiments on the permeability, hygroscopicity and other characters of fruits and seeds, which are likely to affect their chances of dispersal by water; the results of these were published in his *Studies in Seeds and Fruits, an Investigation with the Balance* (1912).

Contemplation of island floras and their probable origin led to views on general distribution and the origin of the families of flowering plants. These views were formulated in a paper read before the Linnean Society in 1918, "Plant Distribution from the Standpoint of an Idealist," in which Guppy maintained that the great families had arisen in an early period of comparatively uniform conditions, and that their differentiation into modern tribes, genera, and species was a response to the differentiation of climatic and other conditions.

Of a singularly modest and retiring disposition, Guppy was rarely seen in places where scientific men congregate, and was probably known to few except by correspondence. Hence perhaps the somewhat tardy recognition of his work. In 1917 the Linnean Society awarded him their medal, and this was followed by his election to the Fellowship of the Royal Society in 1918. He continued his work up to the time of his death, and was returning from Tahiti when he died at Martinique, on the ss. "El Kantara" on April 23 last year, at the age of 71.

A. B. R.

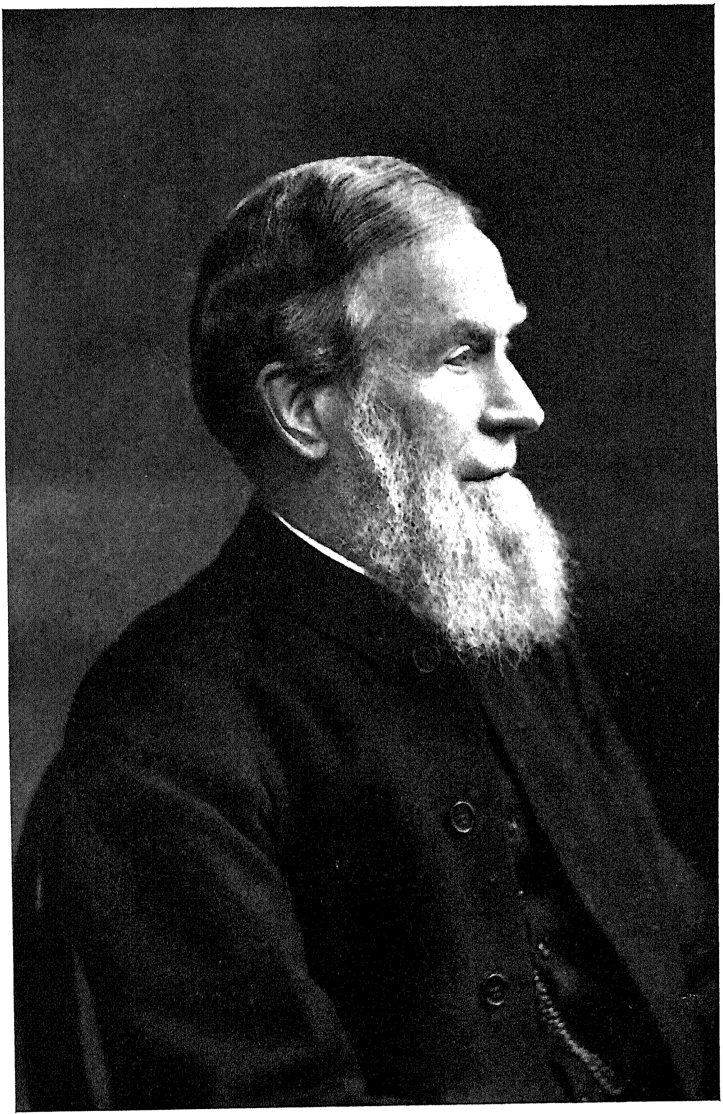
T. R. R. STEBBING—1835–1926.

Thomas Roscoe Rede Stebbing was born in London on February 6, 1835, and died at Tunbridge Wells on July 9, 1926. He was the seventh of the thirteen children of the Reverend Henry Stebbing, D.D., F.R.S., distinguished in his day as a divine and as a man of letters. Several other members of the family inherited their father's literary tastes and abilities. An elder brother, William, who also died in 1926, was for many years on the staff of *The Times*, of which he was sub-editor under Delane.

Thomas Stebbing received his early education at King's College School, whence he went to Oxford, first to Lincoln and later to Worcester College. His academic career was punctuated by prizes and scholarships, and a second-class in *Lit. Hum.* in 1856 was followed by a first in Law and Modern History in 1857. He had taken the B.A. of London in 1855, and in 1859 he was ordained priest by the Bishop of Oxford, Samuel Wilberforce. For the next quarter of a century he was engaged in teaching, at Radley and Wellington, at Worcester College, of which he was fellow, tutor, vice-provost and dean, and latterly with private pupils at Reigate and Torquay.

Up to near the end of this period of his life, Stebbing's studies had been exclusively literary. The first impulse towards Natural History seems to have come from his acquaintance, at Reigate, with W. W. Saunders, F.R.S., the distinguished entomologist and botanist, whose daughter, Mary Anne, he married in 1867. On removing to Torquay, he joined the Torquay Natural History Society and became acquainted with a number of enthusiastic amateurs. One of these was William Pengelly, to whom he afterwards was inclined to give the first place among his "real educators." The way in which Zoology became his main occupation for the remainder of his long life is best told in his own words. The controversy between the representatives of science and those of organised religion was then at its height.

> "Being an enthusiastic young clergyman, and also in these days passionately fond of arguing, I felt it my bounden duty to join in the fray. . . . Accordingly I approached the reading of Charles Darwin's 'Origin of Species' with an easy confidence that I should be able to smash up his heresy and others like it. Instead of which I became an ardent convert, and very soon went on to deliver lectures and preach sermons, harping continuously on the new views. . . . After a while it occurred to me that I knew scarcely anything at first hand of those facts of nature upon which the issue of the contest really rested. This reflection led me to those zoological studies, fascinating but laborious, since pursued through so many years of my life."



Thomas R. R. Stebbing.

The two lines of activity here indicated, the advocacy of a liberal theology and the close study of systematic zoology, together occupied Stebbing down to the last year of his life. He was first brought into prominence as an upholder of the Evolution theory by a letter published in *Nature* of April 20, 1871, commenting on the long and scornful review of the "Descent of Man" which had just appeared in *The Times*. In the same year he published a small volume of "Essays on Darwinism." At that time, it need hardly be said, it was no light matter for a clergyman to declare publicly his adhesion to the new views, and Stebbing incurred not only the suspicion but also the active hostility of his clerical colleagues. He continued, however, to write extensively on this and cognate subjects, and a number of essays contributed to various magazines were afterwards collected in "Faith in Fetters" (1919) and "Plain Speaking" (1926), the latter published only a few months before his death.

The first of Stebbing's original contributions to zoology was a short paper on a fossil coral, *Calceola sandalina* ('Geol. Mag.,' 1873, p. 57), and in the same year he published two short papers on Crustacea, the group of animals which afterwards exclusively occupied his attention. Along with Canon A. M. Norman he began the examination of the Isopoda collected by the "Lightning," "Porcupine" and some other deep-sea expeditions, and a little later, on Canon Norman's recommendation, he was entrusted with the much more important task of working out the Amphipoda of the "Challenger" expedition. About this time he gave up teaching and for some six years devoted all his time and energy to the "Challenger" Amphipoda.

His report, published in 1888, fills three of the ponderous volumes of the "Challenger" series, including 212 plates of figures drawn by the author himself. It is not too much to say that this report set a new standard in systematic carcinology. It dealt with a collection which was probably much larger and was certainly much more varied than any previously brought together. Not only were all the species, new and old, figured, described and compared with a fullness and accuracy of detail rarely attempted before, but the bibliographical introduction which occupies nearly the whole of the first volume provided a critical summary of everything that had been written on the Amphipoda up to the date of its publication. Even to-day there are few groups of invertebrates for which so encyclopædic a guide to the earlier literature exists. The value of this report was not at once recognised in this country, but German zoologists were more appreciative, and the help it gave to later investigators of this group of Crustacea can be traced in their writings.

Eighteen years later Stebbing contributed a revision of the Gammaridea (the largest of the three sections composing the Order Amphipoda) to the series of monographs published by the Berlin Academy of Sciences under the title "Das Tierreich," and in 1910 a smaller volume by him on the Cumacea was included in the same series. These, however, are only the more outstanding of a long series of memoirs that continued to flow from his pen up to the last year of his life. Among the more important might be mentioned many reports on Crustacea of all Orders from South Africa, published in "Marine Investigations in South Africa," edited by the late Dr. J. D. F. Gilchrist, and including a Catalogue of South African Crustacea.

The distinguishing note of Stebbing's carcinological work was that he brought to systematic zoology the spirit and the methods of the older scholarship. Trained exclusively in the schools of classical, theological and legal learning, his writings everywhere show the respect for antiquity, the painstaking collation of authorities and the meticulous verification of references that we associate especially with the word "scholarly."

It is perfectly easy to point out what Stebbing did not do. Taking up the study of zoology only in mature life and without any training in science, he made no contributions of importance to morphology, and he was content, for the most part, to take his systematic categories ready made from others. When he did attempt innovations in classification, as in his revision of the Cumacea, he seemed to prefer a rigid, methodical and practical system of indexing rather than to search for any speculative approach to a natural classification. Stebbing's historical sense and his methodical habit of mind led him to give great attention to problems of nomenclature and to insist strongly on a strict adherence to the principle of priority; but he was by no means inclined to surrender the right of private judgment, or to accept without question the decisions of an international commission.

Stebbing was elected a Fellow of the Royal Society in 1896. He had become a Fellow of the Linnean Society in the preceding year and he repeatedly served on its Council, being a Vice-President in 1902–3 and Zoological Secretary from 1903 to 1907. While holding this office he took a prominent part in the movement which led to the admission of women to the fellowship of the Linnean Society, and his wife, an accomplished botanist, was one of the first group of ladies admitted. Almost at the moment when these words are being written there comes the news that Mrs. Stebbing has survived her husband by only a few months. In 1908 Stebbing was awarded the Linnean medal, an honour which he prized very highly.

Stebbing was of slight physique. His old-fashioned courtesy of manner, kindliness and a certain whimsical humour endeared him to a wide circle of friends. Numerous correspondents, who never saw him, have reason to remember the ready patience with which he allowed the most ignorant to dip into his endless stores of erudition.

W. T. C.

J. N. Langley

JOHN NEWPORT LANGLEY—1852-1925.

On November 5th, 1925, John Newport Langley, Professor of Physiology in the University of Cambridge, died in his seventy-fourth year, after a brief illness, overcome by pneumonia, when he was as strenuously engaged in his own research work and in his regular University duties as at any time during his long and uninterruptedly active life.

Born at Newbury in 1852, the second son of John Langley, a private schoolmaster there, he was educated at home and later at Exeter Grammar School, of which his uncle, the Reverend H. Newport, was Headmaster. Having won an Exhibition at the school, provided by the Acland family of Killerton, Exeter, he proceeded to St. John's College, Cambridge, in October, 1871. He had thoughts of competing later for a place in the Indian Civil Service, and at first his work was aimed at that. He was destined, however, never to leave Cambridge. Here he was to live and work for the rest of his life, and to give more than half a century of unbroken and untiring service to physiology.

It was in his second undergraduate year that he turned from mathematics and history and began to read natural science. This brought him almost at once under the influence and charm of Michael Foster, who had recently been brought from London to Cambridge by the action of Trinity College, on a recommendation the College had sought from Huxley, and who was then conducting classes in Elementary Biology, Embryology and Physiology in a small room lent to him by the University and equipped by the College. It was in May, 1873, that Langley first attended the classes of practical instruction that Foster was then giving, under the newly devised modes that have since spread so widely here and in other countries. He was attracted and held both by teacher and subject, and the course of his life was altered. The first effects of his change of study were soon seen in a new direction of energy and its results. He was elected, in 1874, to a Foundation Scholarship at St. John's College, and in the same year appeared with Milnes Marshall and other friends in the First Class of the Natural Sciences Tripos. In 1875, Foster, who had quickly discerned Langley's powers, appointed him to be his Demonstrator, in succession to Newell Martin, who had left Cambridge for Baltimore, carrying with him there the influence of Foster's ideals and methods, which through him came to have lasting influence in medical education throughout the United States and Canada. In 1877, Langley won a Fellowship at Trinity College, which had been thrown open to men of other Colleges. Thenceforward, Trinity was his home for the fifty years of his remaining life.

As Foster's Demonstrator, from 1875 until 1884, Langley was responsible for the organisation and conduct of all the laboratory class work, while the number of students in physiology was mounting from units to scores. Even before taking his degree he had begun, at Foster's suggestion, a study of the action of jaborandi (pilocarpine) on the heart. The results were given in his first published paper, read before the Cambridge Philosophical Society in April, 1875. This was reprinted in the *Studies from the Physiological Laboratory in the University of Cambridge* for 1876, of which three parts were published, in 1873, 1876 and 1877. Here his work appears side by side with papers by F. M. Balfour, Milnes Marshall, Newell Martin, Dew Smith, S. H. Vines and W. H. Gaskell, who were the chief members of that brilliant band of young workers under Foster's leadership and inspiration to which he was now joined, each one of them destined for work of enduring value. Those published studies, in most parts collected from various Journals, appeared as edited by "The Trinity Prælector in Physiology," under grey covers, bearing the arms of that College.

From work thus begun, Langley, as after events were to show, never turned aside. He decided not to break the thread of his research work by seeking any medical qualification, a decision that suggests many interesting, but perhaps idle, speculations—nor did he ever, even in later years, allow administrative or public work to encroach noticeably upon his unresting course of investigation and teaching. The story of his life is the story of his researches. The outward events marking its stages are easily told. He served under Foster in one position or another until the close of the century. He was his Demonstrator from 1875 until 1884, when he was appointed by Trinity to the permanent College staff as Lecturer in Natural Science and by the University as Lecturer in Histology. Those posts he held until 1903, when he was elected Professor of Physiology in the University, upon Foster's resignation of the Chair, having served already as deputy Professor from 1900. From 1875 to 1900 he had lectured in each of three terms every year and held classes for advanced students in histology. From 1900 for exactly another quarter of a century he lectured to elementary students, first as Deputy Professor, then as Professor, at least three days a week in every term, and gave demonstrations to them, in addition to courses of advanced teaching and in addition to his other work as head of a great school.

The main achievements of Langley's research work stand permanently in their place, not merely as additions made here and there to knowledge, but as indispensable stepping stones along which, at this point or that, the progress of knowledge has actually taken its way. Of all the natural phenomena he examined and described, of all the experimental results he recorded in his long career, it may well be doubted if any subsequent worker using later methods has found occasion to correct or modify the evidence on points of fact which he found and attested and marshalled. All his chief works keep, and must always keep, their place in the significant history of animal physiology. His record of published work is a remarkable one. For just fifty years, from youth to age, he maintained his output of new work. If it could be printed here, the bare list of the titles of his papers and books set out along those years would give the simplest testimony to his unwearying, unhalting, service to science. No single year in all that half-century appears without its contribution of effective work from his own mind and hand in the laboratory.

In broad outline his contributions to physiology may be considered as in two main groups, those dealing with the mechanism of secretion, and those establishing the main anatomical and functional lines of the autonomic nervous system. The former belong to his first fifteen years of work, the latter to the succeeding thirty and more. Chance, as it seems, gave the starting point. Foster, in 1874, suggested his examining the newly-introduced drug, jaborandi (pilocarpine), and its effects on the heart. This he did for frogs and for mammals. This led directly to study of its effects upon secretion, first in the sub-maxillary gland of the dog, and to the researches into the physiology of secretion which followed in close succession until 1890. It was in this earlier work that he exhibited and developed his salient qualities. A merely competent worker might have described the action of pilocarpine, and then have passed, even usefully, to the actions of similar and different drugs. Langley, however, opened up at every point the physiology of secretion, adapting his technical work not to convenience but to his intellectual needs. He made histological studies of gland structure, in activity and rest, checking the interpretation of the appearances of killed and stained cells by simple, but most effective, direct observation of the living gland cells. These were correlated with the experimental unravelling of the nervous influences upon glands, and the share in these taken by the vaso-motor phenomena. Both sets of results were linked again by chemical estimations of the changing qualities of the secretion. The well-known steps he took and the variety of work done are to be traced in his successive papers. Before his work the accepted view had been that of Heidenhain, that gland cells became more granular as secretion took place. Langley showed that the reverse was true; that granules were stored up during rest, to be passed out in secretion, not only in the pancreas, as Kühne and Lea had shown, but over a wide range of glands. By his study of the secretory nerves Langley, moreover, showed that the belief in the existence of special "trophic" or secretory fibres rested on evidence which could be explained in terms of purely vaso-motor changes in the gland circulation. His results, whether finally decisive or not, still hold the field, so far as that argument is concerned, and must in any case always have permanent value of their own. The chief results of his work upon secretory glands were gathered up and incorporated in the valuable article he contributed upon the "Salivary Glands" to Schäfer's 'Text-Book of Physiology,' 1898.

By 1890 Langley had passed on to the next and most conspicuous phase of his work. It was natural that the experimental studies of sympathetic and cranial nerve fibres to the salivary glands should have led him to use the same technical methods in the exploration of other functions of those systems of nerve fibres. To this two other factors contributed. In the years from 1886 to 1889 Gaskell, senior by five years to Langley, had already made his great simplification of bringing into one system, for which he used the word "visceral," not only the nerve fibres of the sympathetic system, which, as he showed, spring from the thoracic and upper lumbar regions, but also those from the cranial or from sacral nerve roots. All these alike, at least in their efferent fibres, are small medullated fibres, and may be considered as one system, interrupted at two points along the axis of the central nervous system by the outflows of fibres, "somatic" as opposed to "visceral," for the arm and for the leg. The cranial and sacral fibres were apparently alike in being antagonistic to the sympathetic fibres.

Gaskell's generalisations, fundamental and illuminating as they were, left very many essential points of anatomical and physiological importance unsettled, and many of these were just of the kind which Langley was already confronting in his own work, and for which both his technical equipment and his temperament alike fitted him. The second factor was the accidental opportunity of trying the effects of another new drug. Just as pilocarpine had led to his studies of secretion, so now a supply of pituri (nicotine) put into his hands by Prof. Liversidge, gave him, as he soon found, a novel and potent instrument, used as he used it, for unravelling the structure and functions of the sympathetic and of the other two parts of the "visceral" or "autonomic" system.

So began that long series of well-known studies, seen in perspective as the eye scans in a list the titles of papers that pass in opulent procession through the years. They deal intensively with the details of the autonomic system for some fifteen years after 1890, and thereafter pick up and follow particular lines of inquiry at some point from the main stem.

There has been an occasional tendency in some writers of recent years not, indeed, to belittle the value of Langley's additions to knowledge in this field, for at almost every point those stand unshaken and secure, but to represent them as counting for not more than the completion of the details of a scheme of which Gaskell had conceived the framework and had laid down the fundamental principles. Gaskell's important advances in this field were made, as already mentioned, between 1886 and 1889. Thereafter he passed to his well-known studies of the origin of vertebrate animals. It is true that by his work on the visceral nervous system, just as by his investigations of the heart and of the heart-beat, Gaskell had given a new orientation to ideas and a new basis for future work. But no more in the one instance than in the other is it right to suggest that the structure of later knowledge, built up in the case of the visceral nervous system so largely by Langley's masterly skill and tireless industry, lay implicit in the foundations so firmly traced by Gaskell. To some extent the suggestion is based, perhaps, on a misreading of Gaskell's own book on the 'Involuntary Nervous System,' published in 1916. In this he gives a summary of the knowledge available at that date, following an account of his own early work up to 1889; the treatment was not intended to be historical and gave no occasion for drawing distinctions between views justified in 1889 and those taken later. As Langley himself wrote in the cordial and well-informed tribute which he paid to Gaskell's work on his death ('Roy. Soc. Proc.,' B, vol. 88, p. xxxi (1915)), "Gaskell's work clarified the air. It gave anatomists and physiologists a clearer view of the general arrangement of the efferent nerves governing unstriated muscle and glands, and it directed the attention of physiologists to points which they had singularly neglected."

When Langley turned his own work in this direction in 1890, our knowledge of the sympathetic system, in spite of Gaskell's inspiring contributions, was still in much dark confusion. It was believed that its efferent fibres might have one or many nerve cells, or none, on their course. By experimental work with nicotine, by degeneration experiments and by histological observation, Langley acquired such multiplied data that we may now believe there is one nerve cell, and one alone, between the central nervous system and the periphery for each nerve fibre. The posterior root ganglia were held to contain visceral efferent as well as sensory nerve cells; that was shown not to be so, and sensory and motor nerve cells were shown not to occur in the same ganglia. It was believed by many, especially by French workers, that true reflex actions could be obtained from peripheral ganglia; Langley showed that the facts could be explained by his "axon-reflexes." By investigating the pilomotor fibres he worked out the segmental distribution of the sympathetic fibres to the skin and their relation to the sensory fibres of the corresponding spinal nerves. There was no general plan of the distribution of nerve fibres of the white rami to the successive ganglia, and this Langley effectively made. Gaskell's fruitful conception of the visceral or autonomic system as a single system of uniform origin, interrupted only by the limb centres, has been modified essentially. To the newer conception of the cranial and sacral outflows of visceral nerve-fibres and their connected ganglia, as belonging to a system, which Langley called "oro-anal," different in origin, distribution and function from the thoracico-lumbar sympathetic system, Langley's physiological analysis contributed, in harmony with Gaskell's later and chiefly morphological work. These studies of the automonic system by Langley, together with the earlier studies of Gaskell, to which they provided exactly the complement required, must always remain among the greatest of the achievements of English physiology.

Important as were the broad generalisations thus established or enriched by Langley's work, tribute must be paid to the great beauty and interest of very many of the individual researches within the series. His studies with H. K. Anderson, now Master of Caius, of the nervous and muscular mechanisms of the iris ; his demonstrations of the pilo-motor mechanisms and their equivalents for the feathers of birds ; his unravelling, again with Anderson, of the intricate nervous supplies to the various pelvic organs ; his proof of the general functional similarity between all efferent nerve-fibres by successful cross-unions of bulbar-autonomic, of sympathetic and of somatic nerves, after which each was shown to take on after regeneration the functions of another ; of these or of many other researches each physiologist will make his own choice for special admiration.

From 1905 onwards Langley turned close attention to the mode of functional union between nerve-fibre and muscle substance. Here he gave another extension to his analytic use of drugs in experiments. Curari and similar drugs had been generally held to exert their actions upon the last terminal branches of the nerve-fibre and to be without action on the tissue in which the fibre ended. Langley had shown that nicotine stimulates peripheral nerve-cells after degeneration of the preganglionic fibres ending in them. In the peripheral tissues the persistence of the action of adrenaline, which had been demonstrated by Levandowsky, and by himself, after degeneration of the sympathetic fibres, made its point of action appear to be a hypothetical neuro-muscular "junction," as Elliott had concluded already from his work at the same time in the Cambridge laboratory. Langley found that nicotine applied directly to certain muscles gave a contraction limited to the area just under or near the nerve-ending, and that this localised contraction was prevented by curari. These and other observations led him to suppose that the region just under a nerve-ending, which he called the "neural region," is specially excitable, probably because "receptive substances" are there, or are liberated there. He concluded that the specific action of most poisons depends on the nature of the receptive substance present, while some poisons may have in addition their own action upon the actual end branches of the nerve-fibre itself. His results in this field of work are given in his Croonian Lecture before the Royal Society in 1906, and received further development in the years following.

During the first decade of this century, Langley was leading an active school of workers, fast growing beyond the bounds for which Foster had provided when, in 1890, crowded out of his first physiological laboratory, he had very greatly extended and enlarged it. The building was already inadequate for the increasing numbers of students and it was almost ludicrously inadequate for the needs of the research work being done in many different fields of physiology and biochemistry by the various members of Langley's staff and their juniors. Of Langley's near contemporaries, Sheridan Lea had gone and only Gaskell remained, adding his constant contributions of help and inspiration. A congeries of small and badly-arranged research rooms was filled, and over-filled, by the workers for whom Langley had to contrive accommodation. "Accommodation" was even provided by pressing a disused coal-cellar into the service of three workers. One small research room served for biochemistry, and for the whole laboratory there was only one accurate chemical balance. Those were the days before Government help or private munificence to physiology and its companions had seriously begun. To name only those who became Fellows of this Society, there were in those years at work here, together with Gaskell and Langley, and almost all of them simultaneously, Hardy, Anderson, Rivers, Hopkins, Elliot-Smith, Fletcher, Barcroft, Dale, Elliott, Lucas, A. V. Hill and Adrian, besides many others. In 1910, the Drapers' Company of London offered to build a new School of Physiology at Cambridge: the University accepted this and before long provided a new site. Langley gave close attention to the design and equipment of the new School as it was being planned, and it was formally opened by Prince Arthur of Connaught in June, 1914.

The provision of this great building was a fitting recognition of the distinction and activity of the school of workers which Langley was leading; for the first time they were to have reasonable space and equipment for investigation, and it was with high hopes that Langley passed over with them to its occupation. Within a few weeks, however, the War began. In coming it brought relative emptiness to the building; it was to bring also irreparable personal losses; but it was to prove to be only a postponement of hope and achievement.

During the War, Langley turned his own studies into directions likely to have medical value. He investigated in particular the changes in muscle after section of the motor nerves, the modes in which atrophy followed and the effects upon it of massage and electrical stimulation. All his junior colleagues were upon other forms of war service, but he was assisted in these, and in other studies of nervous degeneration and of the anatomy of nerve trunks, by various Japanese workers. In the succeeding years, as the tide of students returned after the War and colleagues were regained, he turned to gathering up and supplementing his main body of work upon the autonomic system, and he prepared his book *The Autonomic Nervous System*, of which Part I was published in 1921. This was soon translated into both French and German. In the last years he studied again the secretory activity of skin glands, and published a series of papers upon the capillary circulation and its nervous control in vascular reflexes. On the day before his last short illness he was engaged in a long experiment which kept him busily and continuously at work for six hours. In this he seemed to his laboratory assistant, who had helped him for nearly forty years, to be as strenuous in concentrated observation and as skilful in his handiwork as at any time during that long tale of work.

Langley's investigations gained early recognition and many appropriate honours came to him. In 1883 he was elected into the Royal Society, at the age of 31. In 1892 he received a Royal Medal from the Society. He served upon the Council for two periods and was Vice-President in 1904-5. In 1893 he was President of the Neurological Society of Great Britain. He received the Baly Medal of the Royal College of Physicians in 1903, and in 1912 the Retzius Medal of the Swedish Society of Physicians. Honorary degrees were conferred on him at Dublin, St. Andrew's, Groningen and Strasbourg, and he was honorary member of almost every important academy of physiology or medicine in other countries.

We must not omit to notice here an important part of Langley's life-work in which he gave service of incalculable value to physiology both in this country and in others. Beyond all his work as a teacher and leader in Cambridge, and beyond his maintained labours as investigator, Langley undertook the heavy and unremitting task of editing the *Journal of Physiology* for more than thirty years, and he so interpreted and performed his duty in that respect as to bring aid, seen or unseen, to other workers all over the world. The *Journal* was founded by Foster in 1878. In 1894, it was becoming increasingly in debt and was threatened with extinction. Langley in that year, arranged to pay off the debt, a considerable sum, and to receive the unsold stock. Thenceforward and until his death he owned and edited the *Journal*, though Foster's name was retained upon the cover during his lifetime. The remuneration he earned as owner of the *Journal* was small recompense for his heavy labours as editor.

In the midst of the work of teaching and research already described, work which would have filled more than a lifetime for most men, he bore continuously this extra burden of reading, of editorial correspondence, and of press management. Unlike most editors, for him the acceptance of a paper meant often not the end, but the beginning of a task. He thought it due to the *Journal* and to science that the paper should be cast in the most effective form and reduced, compatibly with that, to the least size. This meant correction, often heavy correction, commonly suggested in detail by himself, and it involved the exchange of views with sensitive and sometimes irascible authors. Not a few workers have been surprised to receive their paper again in manuscript almost entirely re-written by Langley. Those to whom this may have come as an unwelcome shock were those who had most cause of gratitude for the service done them. On this side of the work he often showed signal generosity. For younger men, and especially for foreign contributors, he would often completely recast a paper, amend or extend references, revise tables, draft conclusions, and would accept no published acknowledgment. At the least it may be said that if he had difficulties with an occasional contributor, the immense majority were well served and grateful, while Langley put the whole body of workers using the *Journal* into his lasting debt. He made, and he has now left, the *Journal of Physiology* unsurpassed in the high standards maintained, both as to the content and as to the form of the papers within it.

Even to the oldest of his living pupils, Langley seemed to remain always at the same age and in the same wiry health. He was spare and upright in figure, alert and trim in his carriage. The passing years seemed to bring no change to his brisk step or to his appearance, save for some late greying of his hair. The firm lines of his face and the vivid steel-blue of his notable eyes seemed to stay quite untouched by time. He had no professorial manner; he cared for the position, but not for the title of professor, and never indulged in the solemnity of office. To all his pupils, old or young, as to his own friends, he was ready always to show a genial interest in their concerns, warmed with a smile that tempered the first effects of his arresting and observant eyes. It was in private talk and informal demonstrations that he was at his best as a teacher, In formal lecturing, he was not so effective, for he was too critical of himself and of his subject matter to be able to use in public, as he might in private, the selective and emphatic dogmatism that successful elementary teaching requires. His chief and lasting influence upon his senior pupils was by the example of his own work and by direct precept in the technical directions in which he was a master craftsman.

He had many strong interests in outdoor games and sports, and maintained his bodily vigour to the end of his life. In early days he was a good runner and was fond of long rowing and sailing excursions. Throughout his middle age he played tennis, lawn tennis and golf, as actively as the claims of his work allowed. He was a skilful and enthusiastic skater and excelled in all the intricacies of the English style of skating, whenever he had time for a short winter holiday in Switzerland or found opportunities at home.

In 1902 he married Vera Kathleen, daughter of Frederick Forsyth-Grant of Ecclesgreig, Kincardineshire. He left his College rooms to live in a house with a large garden on the outskirts of Cambridge, towards Madingley, and here his daughter, Alison, was born. His marriage brought him great happiness. His garden provided him with a hobby, from which he gained much in health as more active exercises were laid aside. Like Foster, and like Gaskell, Langley was now to find gardening his chief preoccupation outside his daily work, and like them he showed great skill both in planning and in cultivation. His tulips in May and his roses in June were unsurpassed in Cambridge, where good gardeners abound. Here he happily filled out the span of his life and here he died in the full harness of active work, painlessly and speedily. He had seemed never to grow old and he had known no weakness or decrepitude. He passed from his work leaving a proud record of long and tireless service to knowledge.

W. M. F.

ERRATA.

Vol. 100 (B), 1926.

In the Obituary Notice of Sir Frederick Mott, p. xxviii :—

In title. *For* 1855, *read* 1853.

Line 1. *For* Walter, *read* Walker.
For June 9, *read* June 8.

Line 10 from bottom. *After* was appointed, *insert* (in 1883).

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